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(54) Title: HYBRID ADENOVIRUS-AAV VIRUS AND METHOD OF USE THEREOF

(57) Abstract

The present invention provides a hybrid vector construct which comprises a portion of an adenovirus, 5' and 3' ITR sequences from an AAV, and a selected transgene. Also provided is a hybrid virus linked via a polycation conjugate to an AAV *rep* gene to form a single particle. These trans-infection particles are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome. Also disclosed is the use of the hybrid vectors and viruses to produce large quantities of recombinant AAV.

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HYBRID ADENOVIRUS-AAV VIRUS AND METHODS OF USE THEREOF

This invention was supported by the National
Institute of Health Grant No. P30 DK 47757. The United
5   States government has rights in this invention.

Field of the Invention

The present invention relates to the field of
vectors useful in somatic gene therapy and the production
10 thereof.

Background of the Invention

Recombinant adenoviruses are capable of
providing extremely high levels of transgene delivery to
15 virtually all cell types, regardless of the mitotic
state. High titers (10^{13} plaque forming units/ml) of
recombinant virus can be easily generated in 293 cells
(the adenovirus equivalent to retrovirus packaging cell
lines) and cryo-stored for extended periods without
20 appreciable losses.

The primary limitation of this virus as a
vector resides in the complexity of the adenovirus
genome. A human adenovirus is comprised of a linear,
approximately 36 kb double-stranded DNA genome, which is
25 divided into 100 map units (m.u.), each of which is 360
bp in length. The DNA contains short inverted terminal
repeats (ITR) at each end of the genome that are required
for viral DNA replication. The gene products are
organized into early (E1 through E4) and late (L1 through
30 L5) regions, based on expression before or after the
initiation of viral DNA synthesis [see, e.g., Horwitz,
Virology, 2d edit., ed. B. N. Fields, Raven Press, Ltd. ,
New York (1990)].

A human adenovirus undergoes a highly regulated
35 program during its normal viral life cycle [Y. Yang et
al, Proc. Natl. Acad. Sci., USA, 91:4407-4411 (1994)].

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virions are internalized by receptor-mediated endocytosis
and transported to the nucleus where the immediate early
genes, Ela and Elb, are expressed. Because these early
gene products regulate expression of a variety of host
5   genes (which prime the cell for virus production) and are
central to the cascade activation of early delayed genes
(e.g. E2, E3, and E4) followed by late genes (e.g. L1-5),
first generation recombinant adenoviruses for gene
therapy focused on the removal of the E1 domain. This
10 strategy was successful in rendering the vectors
replication defective, however, *in vivo* studies revealed
transgene expression was transient and invariably
associated with the development of severe inflammation at
the site of vector targeting [S. Ishibashi et al, <u>J.</u>
15 <u>Clin. Invest.</u>, <u>93</u>:1885-1893 (1994); J. M. Wilson et al,
<u>Proc. Natl. Acad. Sci., USA</u>, <u>85</u>:4421-4424 (1988); J. M.
Wilson et al, <u>Clin. Bio.</u>, <u>3</u>:21-26 (1991); M. Grossman et
al, <u>Som. Cell. and Mol. Gen.</u>, <u>17</u>:601-607 (1991)].
       Adeno-associated viruses (AAV) have also been
20 employed as vectors. AAV is a small, single-stranded
(ss) DNA virus with a simple genomic organization (4.7
kb) that makes it an ideal substrate for genetic
engineering. Two open reading frames encode a series of
rep and *cap* polypeptides. *Rep* polypeptides (*rep78*,
25 *rep68*, *rep62* and *rep40*) are involved in replication,
rescue and integration of the AAV genome. The *cap*
proteins (VP1, VP2 and VP3) form the virion capsid.
Flanking the *rep* and *cap* open reading frames at the 5'
and 3' ends are 145 bp inverted terminal repeats (ITRs),
30 the first 125 bp of which are capable of forming Y- or T-
shaped duplex structures. Of importance for the
d velopment of AAV vectors, the entire *r p* and *cap*
domains can be excised and replac d with a therapeutic or
reporter transg ne [B. J. Cart r, in "Handbook of
35 Parvovirus s", ed., P. Tijss r, CRC Press, pp.155-168

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(1990)]. It has been shown that the ITRs represent the
minimal sequence required for replication, rescue,
packaging, and integration of the AAV genome.

5   The AAV life cycle is biphasic, composed of
both latent and lytic episodes. During a latent
infection, AAV virions enter a cell as an encapsidated
ssDNA, and shortly thereafter are delivered to the
nucleus where the AAV DNA stably integrates into a host
chromosome without the apparent need for host cell

10   division. In the absence of helper virus, the integrated
ss DNA AAV genome remains latent but capable of being
activated and rescued. The lytic phase of the life cycle
begins when a cell harboring an AAV provirus is
challenged with a secondary infection by a herpesvirus or

15   adenovirus which encodes helper functions that are
recruited by AAV to aid in its excision from host
chromatin [B. J. Carter, cited above]. The infecting
parental ssDNA is expanded to duplex replicating form
(RF) DNAs in a *rep* dependent manner. The rescued AAV

20   genomes are packaged into preformed protein capsids
(icosahedral symmetry approximately 20 nm in diameter)
and released as infectious virions that have packaged
either + or - ss DNA genomes following cell lysis.

Progress towards establishing AAV as a

25   transducing vector for gene therapy has been slow for a
variety of reasons. While the ability of AAV to
integrate in quiescent cells is important in terms of
long term expression of a potential transducing gene, the
tendency of the integrated provirus to preferentially

30   target only specific sites in chromosome 19 reduces its
usefulness. Additionally, difficulties surround large-
scale production of replication defectiv recombinants.
In contrast to the production of recombinant retrovirus
or adenovirus, the only widely recognized means for

35   manufacturing transducing AAV virions entails co-

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transfection with two different, yet complementing
plasmids. One of these contains the therapeutic or
reporter minigene sandwiched between the two *cis* acting
AAV ITRs. The AAV components that are needed for rescue
5   and subsequent packaging of progeny recombinant genomes
are provided in *trans* by a second plasmid encoding the
viral open reading frames for *rep* and *cap* proteins. The
cells targeted for transfection must also be infected
with adenovirus thus providing the necessary helper
10   functions. Because the yield of recombinant AAV is
dependent on the number of cells that are transfected
with the *cis* and *trans*-acting plasmids, it is desirable
to use a transfection protocol with high efficiency. For
large-scale production of high titer virus, however,
15   previously employed high efficiency calcium phosphate and
liposome systems are cumbersome and subject to
inconsistencies.
         There remains a need in the art for the
development of vectors which overcome the disadvantages
20   of the known vector systems.

Summary of the Invention
         In one aspect, the present invention provides a
unique recombinant hybrid adenovirus/AAV virus, which
25   comprises an adenovirus capsid containing selected
portions of an adenovirus sequence, 5' and 3' AAV ITR
sequences which flank a selected transgene under the
control of a selected promoter and other conventional
vector regulatory components. This hybrid virus is
30   characterized by high titer transgene delivery to a host
cell and the ability to stably integrate the transgene
into the host cell chromosome in the presence of the *rep*
gene. In one embodiment, th transgene is a reporter
gene. Another embodim nt of the hybrid virus contains a
35   therapeutic transgene. In a pr ferred embodiment, the

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hybrid virus has associated therewith a polycation
sequence and the AAV *rep* gene. This construct is termed
the hybrid virus conjugate or trans-infection particle.

In another aspect, the present invention
provides a hybrid vector construct for use in producing
the hybrid virus or viral particle described above. This
hybrid vector comprises selected portions of an
adenovirus sequence, 5' and 3' AAV ITR sequences which
flank a selected transgene under the control of a
selected promoter and other conventional vector
regulatory components.

In another aspect, the invention provides a
composition comprising a hybrid viral particle for use in
delivering a selected gene to a host cell. Such a
composition may be employed to deliver a therapeutic gene
to a targeted host cell to treat or correct a genetically
associated disorder or disease.

In yet another aspect, the present invention
provides a method for producing the hybrid virus by
transfecting a suitable packaging cell line with the
hybrid vector construct of this invention. In another
embodiment the method involves co-transfecting a cell
line (either a packaging cell line or a non-packaging
cell line) with a hybrid vector construct and a suitable
helper virus.

In a further aspect, the present invention
provides a method for producing large quantities of
recombinant AAV particles with high efficiency by
employing the above methods, employing the hybrid vector
construct of this invention and collecting the rAAV
particles from a packaging cell line transfected with the
vector.

Other aspects and advantages of the pres nt
invention are d scribed further in the following detailed
d scription of the preferred mbodiments thereof.

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Brief Description of the Drawings

Fig. 1A is a schematic diagram of a vector construct pAd.AV.CMVLacZ [SEQ ID NO: 1], which contains (from the top in clockwise order) adenovirus sequence map units 0-1 (clear bar); the 5' AAV ITR (solid bar); a CMV immediate early enhancer/promoter (hatched arrow), an SV40 intron (clear bar), an E. coli beta-galactosidase cDNA (LacZ) (hatched line), an SV40 polyadenylation signal (clear bar), a 3' AAV ITR (solid bar), adenovirus sequence from map units 9-16 (clear bar), and a portion of a pBR322 derivative plasmid (thin solid line). Restriction endonuclease enzymes are identified by their conventional designations; and the location of each restriction enzyme is identification by the nucleotide number in parentheses to the right of the enzyme designation.

Fig. 1B is a schematic drawing demonstrating linearization of pAd.AV.CMVLacZ [SEQ ID NO: 1] by digestion with restriction enzyme NheI and a linear arrangement of a ClaI digested adenovirus type 5 with deletions from mu 0-1. The area where homologous recombination will occur (between m.u. 9-16) in both the plasmid and adenovirus sequences is indicated by crossed lines.

Fig. 1C is a schematic drawing which demonstrates the hybrid virus Ad.AV.CMVLacZ after co-transfection of the linearized pAd.AV.CMVLacZ [SEQ ID NO: 1] and adenovirus into 293 cells followed by intracellular homologous recombination.

Fig. 2A-2K report the top DNA strand of the double-strand plasmid pAd.AV.CMVLacZ [SEQ ID NO: 1] (the compl mentary strand can be readily d rived by one of skill in the art). With reference to SEQ ID NO: 1, nucleotides 1-365 ar ad novirus type 5 s quences; the 5' AAV ITR sequenc spans nucleotides 366-538; the CMV

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promoter/enhancer spans nucleotides 563-1157; the SV-40
intron spans nucleotides 1158-1179; the *LacZ* gene spans
nucleotides 1356-4827; the SV-40 poly A sequence spans
nucleotides 4839-5037; the 3' AAV ITR spans nucleotides

5   5053 to 5221; nucleotides 5221 to about 8100 are
adenovirus type 5 sequences. The remaining sequences are
non-specific/plasmid sequences.

Fig. 3 is a bar graph plotting u.v. absorbance
at 420 nm of the beta-galactosidase blue color for a

10   control and ten putative positive clones (D1A through
D1J) of 293 cells transfected with the recombinant hybrid
Ad.AV.CMV*LacZ*. Eight of the clones expressed high levels
of enzyme.

Fig. 4 is a schematic diagram of pRep78/52 [SEQ

15   ID NO: 2]. This plasmid includes an AAV P5 promoter,
Rep78, Rep52 and a poly-A sequence in a pUC18 plasmid
background.

Figs. 5A - 5E report nucleotides 1-4910 of the
top DNA strand of the double-strand plasmid pRep78/52

20   [SEQ ID NO: 2] (the complementary strand can be readily
derived by one of skill in the art).

Fig. 6 is a flow diagram of the construction of
a trans-infection particle formed by a hybrid virus, a
poly-L-lysine sequence and attached AAV *rep*-containing

25   plasmid.

Fig. 7 is a flow diagram of the hybrid virus'
life cycle, in which a trans-infection particle enters
the cell and is transported to the nucleus. The virus is
uncoated and the *rep* mediates rescue of the inserted

30   gene, which is then integrated into the chromosome of the
host cell.

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(57) Abstract

The present invention provides a hybrid vector construct which comprises a portion of an adenovirus, 5' and 3' ITR sequences from an AAV, and a selected transgene. Also provided is a hybrid virus linked via a polycation conjugate to an AAV *rep* gene to form a single particle. These trans-infection particles are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome. Also disclosed is the use of the hybrid vectors and viruses to produce large quantities of recombinant AAV.

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I.   **Construction of the Hybrid Vector and Virus**

A.   _The Adenovirus Component of the Vector and_
Virus

The hybrid virus of this invention uses
5   adenovirus nucleic acid sequences as a shuttle to deliver
a recombinant AAV/transgene genome to a target cell. The
DNA sequences of a number of adenovirus types, including
type Ad5, are available from Genbank. The adenovirus
sequences may be obtained from any known adenovirus type,
10   including the presently identified 41 human types
[Horwitz et al, cited above]. Similarly adenoviruses
known to infect other animals may also be employed in the
vector constructs of this invention. The selection of
the adenovirus type is not anticipated to limit the
15   following invention. A variety of adenovirus strains are
available from the American Type Culture Collection,
Rockville, Maryland, or available by request from a
variety of commercial and institutional sources. In the
following exemplary embodiment an adenovirus, type 5
20   (Ad5) is used for convenience.

The adenovirus nucleic acid sequences
employed in the hybrid vector of this invention can range
from a minimum sequence amount, which requires the use of
a helper virus to produce the hybrid virus particle, to
25   only selected deletions of adenovirus genes, which
deleted gene products can be supplied in the hybrid viral
production process by a selected packaging cell.
Specifically, at a minimum, the adenovirus nucleic acid
sequences employed in the pAdΔ shuttle vector of this
30   invention are adenovirus genomic sequences from which all
viral genes are deleted and which contain only those
adenovirus s quences requir d for packaging adenoviral
genomic DNA into a pref rmed capsid head. More
specifically, the adenovirus s quenc s employed are the
35   cis-acting 5' and 3' inverted t rminal repeat (ITR)

sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter.

5   According to this invention, the entire adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region can be employed as the 5' adenovirus sequence in the hybrid virus. This left terminal (5') sequence of the Ad5 genome useful in this invention spans bp 1 to

10   about 360 of the conventional adenovirus genome, also referred to as map units 0-1 of the viral genome, and generally is from about 353 to about 360 nucleotides in length. This sequence includes the 5' ITR (bp 1-103 of the adenovirus genome); and the packaging/enhancer domain

15   (bp 194-358 of the adenovirus genome). Preferably, this native adenovirus 5' region is employed in the hybrid virus and vector in unmodified form. Alternatively, corresponding sequences from other adenovirus types may be substituted. These Ad sequences may be modified to

20   contain desired deletions, substitutions, or mutations, provided that the desired function is not eliminated.

The 3' adenovirus sequences of the hybrid virus include the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the

25   adenovirus genome, or map units ~98.4-100. This sequence is generally about 580 nucleotide in length. This entire sequence is desirably employed as the 3' sequence of a hybrid virus. Preferably, the native adenovirus 3' region is employed in the hybrid virus in unmodified

30   form. However, as described above with respect to the 5' sequences, some modifications to these sequences which do not adversely eff ct their biological function may be acceptable. As described below, wh n these 5' and 3' adenovirus sequences ar employed in the hybrid vector, a

35   helper adenovirus which supplies all other essential

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genes for viral formation alone or with a packaging cell
line is required in the production of the hybrid virus or
viral particle.

Alternative embodiments of the hybrid
5   virus employ adenovirus sequences in addition to the
minimum sequences, but which contain deletions of all or
portions of adenovirus genes. For example, the
adenovirus immediate early gene E1a (which spans mu 1.3
to 4.5) and delayed early gene E1b (which spans mu 4.6 to
10   11.2) should be deleted from the adenovirus sequence
which forms a part of the hybrid vector construct and
virus. Alternatively, if these sequences are not
completely eliminated, at least a sufficient portion of
the E1a and E1b sequences must be deleted so as to render
15   the virus replication defective. These deletions,
whether complete or partial, which eliminate the
biological function of the gene are termed "functional
deletions" herein.

Additionally, all or a portion of the
20   adenovirus delayed early gene E3 (which spans mu 76.6 to
86.2) may be eliminated from the adenovirus sequence
which forms a part of the hybrid virus. The function of
E3 is irrelevant to the function and production of the
hybrid virus.

25   All or a portion of the adenovirus delayed
early gene E2a (which spans mu 67.9 to 61.5) may be
eliminated from the hybrid virus. It is also anticipated
that portions of the other delayed early genes E2b (which
spans mu 29 to 14.2) and E4 (which spans mu 96.8 to 91.3)
30   may also be eliminated from the hybrid virus and from the
vector.

Deletions may also be made in any of the
late genes L1 through L5, which span mu 16.45 to 99 of
the adenovirus g nome. Similarly, deletions may be
35   useful in the intermediate genes IX which maps between mu

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9.8 and 11.2 and IVa_2 which maps between 16.1 to 11.1.
Other deletions may occur in the other structural or non-
structural adenovirus.

The above discussed deletions may occur
5   individually, i.e., an adenovirus sequence for use in the
present invention may contain deletions of E1 only.
Alternatively, deletions of entire genes or portions
effective to destroy their biological activity may occur
in any combination. For example, in one exemplary hybrid
10 vector, the adenovirus sequence may contain deletions of
the E1 genes and the E3 gene, or of the E1, E2a and E3
genes, or of the E1 and E4 genes, or of E1, E2a and E4
genes, with or without deletion of E3, and so on.

The more deletions in the adenovirus
15 sequence up to the minimum sequences identified above
that characterize the hybrid virus, the larger the
sequence(s) of the other below-described components to be
inserted in the hybrid vector. As described above for
the minimum adenovirus sequences, those gene sequences
20 not present in the adenovirus portion of the hybrid virus
must be supplied by either a packaging cell line and/or a
helper adenovirus to generate the hybrid virus.

In an exemplary hybrid virus of this invention
which is described below and in Example 1, the adenovirus
25 genomic sequences present are from mu 0 to 1, mu 9 to
78.3 and mu 86 to 100 (deleted sequences eliminate the
E1a and E1b genes and a portion of the E3 gene). From
the foregoing information, it is expected that one of
skill in the art may construct hybrid vectors and viruses
30 containing more or less of the adenovirus gene sequence.

The portions of the adenovirus genome in
the hybrid virus permit high production titers of the
virus to be produced, often greater than 1×10^{13} pfu/ml.
This is in stark contrast to the low titers (1×10^6
35 pfu/ml) that have been found for recombinant AAV.

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B.   *The AAV Components of the Vector and Virus*

Also part of the hybrid vectors and viruses of this invention are sequences of an adeno-associated virus. The AAV sequences useful in the hybrid
5 vector are the viral sequences from which the *rep* and *cap* polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses",
10 ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. As stated above, the ITR sequences are about 143 bp in length. Substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be
15 permissible for this use. See, e.g., WO 93/24641, published December 9, 1993. The ability to modify these ITR sequences is within the skill of the art. For suitable techniques, see, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit.,
20 Cold Spring Harbor Laboratory, New York (1989).

The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. Similarly, AAVs known to infect other animals may also be employed in the vector constructs of this
25 invention. The selection of the AAV is not anticipated to limit the following invention. A variety of AAV strains, types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. In the
30 following exemplary embodiment an AAV-2 is used for convenience.

In the hybrid vector construct, the AAV sequ nces ar flanked by the sel cted adenovirus sequences discussed above. The 5' and 3' AAV ITR
35 sequ nces themselves flank a selected transgene sequence

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and associated regulatory elements, described below.
Thus, the sequence formed by the transgene and flanking
5' and 3' AAV sequences may be inserted at any deletion
site in the adenovirus sequences of the vector. For
example, the AAV sequences are desirably inserted at the
site of the deleted E1a/E1b genes of the adenovirus,
i.e., after map unit 1. Alternatively, the AAV sequences
may be inserted at an E3 deletion, E2a deletion, and so
on. If only the adenovirus 5' ITR/packaging sequences
and 3' ITR sequences are used in the hybrid virus, the
AAV sequences are inserted between them.

C. *The Transgene Component of the Hybrid
Vector and Virus*

The transgene sequence of the vector and
recombinant virus is a nucleic acid sequence or reverse
transcript thereof, heterologous to the adenovirus
sequence, which encodes a polypeptide or protein of
interest. The transgene is operatively linked to
regulatory components in a manner which permits transgene
transcription.

The composition of the transgene sequence
will depend upon the use to which the resulting hybrid
vector will be put. For example, one type of transgene
sequence includes a reporter sequence, which upon
expression produces a detectable signal. Such reporter
sequences include without limitation an *E. coli* beta-
galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene
and a green fluorescent protein gene. These sequences,
when associated with regulatory elements which drive
their expression, provide signals detectable by
conventional means, e.g., ultraviolet wavelength
absorbance, visible color change, etc.

Another type of transgene sequence
includes a therapeutic gene which expresses a desired
gene product in a host cell. These therapeutic genes or

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nucleic acid sequences typically encode products for
administration and expression in a patient *in vivo* or *ex
vivo* to replace or correct an inherited or non-inherited
genetic defect or treat an epigenetic disorder or

5   disease. Such therapeutic geneᵇ which are desirable for
the performance of gene therapy include, without
limitation, a normal cystic fibrosis transmembrane
regulator (CFTR) gene, a low density lipoprotein (LDL)
gene, and a number of genes which may be readily selected

10   by one of skill in the art. The selection of the
transgene is not considered to be a limitation of this
invention, as such selection is within the knowledge of
those skilled in the art.

        D.   *Regulatory Elements of the Hybrid Vector*

15   In addition to the major elements
identified above for the hybrid vector, i.e., the
adenovirus sequences, AAV sequences and the transgene,
the vector also includes conventional regulatory elements
necessary to drive expression of the transgene in a cell

20   transfected with the hybrid vector. Thus the vector
contains a selected promoter which is linked to the
transgene and located, with the transgene, between the
AAV ITR sequences of the vector.

                Selection of the promoter is a routine

25   matter and is not a limitation of the hybrid vector
itself. Useful promoters may be constitutive promoters
or regulated (inducible) promoters, which will enable
control of the amount of the transgene to be expressed.
For example, a desirable promoter is that of the

30   cytomegalovirus immediate early promoter/enhancer [see,
e.g., Boshart et al, Cell, 41:521-530 (1985)]. Other
desirable promot rs include, without limitation, the Rous
sarcoma virus LTR promoter/enhancer and the chicken β-
actin promoter. Still other promoter/enhancer sequences

35   may be selected by one f skill in the art.

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The vectors will also desirably contain nucleic acid sequences heterologous to the adenovirus sequences including sequences providing signals required for efficient polyadenylation of the transcript and

5 introns with functional splice dunor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted in the vector following the transgene sequences

10 and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. A hybrid vector of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence

15 and the transgene. Selection of these and other common vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein]. The DNA sequences encoding such regulatory regions are provided in the plasmid sequence

20 of Fig. 2 [SEQ ID NO: 1].

The combination of the transgene, promoter/enhancer, the other regulatory vector elements and the flanking 5' and 3' AAV ITRs are referred to as a "minigene" for ease of reference herein. As above

25 stated, the minigene is located in the site of any selected adenovirus deletion in the hybrid virus. The size of this minigene depends upon the amount and number of adenovirus sequence deletions referred to above. Such a minigene may be about 8 kb in size in the exemplary

30 virus deleted in the E1 and E3 genes, as described in the examples below. Alternatively, if only the minimum adenovirus sequenc s are employed in the virus, this minigene may be a size up to about 30 kb. Thus, this hybrid vector and vector p rmit a gr at deal of latitude

35 in the selection of the various components of the

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minigene, particularly the transgene, with regard to
size. Provided with the teachings of this invention, the
design of such a minigene can be made by resort to
conventional techniques.

5   E.   *Hybrid Vector Assembly and Production of*
Hybrid Virus

The material from which the sequences used
in the hybrid vector, helper viruses, if needed, and
recombinant hybrid virus (or viral particle) are derived
10   and the various vector components and sequences employed
in the construction of the hybrid vectors of this
invention are obtained from commercial or academic
sources based on previously published and described
materials. These materials may also be obtained from an
15   individual patient or generated and selected using
standard recombinant molecular cloning techniques known
and practiced by those skilled in the art. Any
modification of existing nucleic acid sequences forming
the vectors and viruses, including sequence deletions,
20   insertions, and other mutations are also generated using
standard techniques.

Assembly of the selected DNA sequences of
the adenovirus, the AAV and the reporter genes or
therapeutic genes and other vector elements into the
25   hybrid vector and the use of the hybrid vector to produce
a hybrid virus utilize conventional techniques, such as
described in Example 1. Such techniques include
conventional cloning techniques of cDNA such as those
described in texts [Sambrook et al, cited above], use of
30   overlapping oligonucleotide sequences of the adenovirus
and AAV genomes, polymerase chain reaction, and any
suitable method which provides the desired nucleotide
sequence. Standard transfction and co-transfection
techniques are employ d, e.g., CaPO$_4$ transfection
35   techniques using the complementation human embryonic

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kidney (HEK) 293 cell line (a human kidney cell line
containing a functional adenovirus E1a gene which
provides a transacting E1a protein). Other conventional
methods employed in this invention include homologous
5   recombination of the viral genomes, plaquing of viruses
in agar overlay, methods of measuring signal generation,
and the like.

As described in detail in Example 1 below
and with resort to Fig. 1, a unique hybrid virus of this
10   invention is prepared which contains an E1-deleted,
partially E3 deleted, adenovirus sequence associated with
a single copy of a recombinant AAV having deletions of
its *rep* and *cap* genes and encoding a selected reporter
transgene. Briefly, this exemplary hybrid virus was
15   designed such that the AV.CMV*LacZ* sequence [SEQ ID NO: 1]
(a minigene containing a 5'AAV ITR, a CMV promoter, an
SV-40 intron, a *LacZ* transgene, an SV-40 poly-A sequence
and a 3' AAV ITR) was positioned in place of the
adenovirus type 5 (Ad5) E1a/E1b genes, making the
20   adenovirus vector replication defective.

Because of the limited amount of
adenovirus sequence present in the hybrid vectors of this
invention, including the pAV.CMV*LacZ* [SEQ ID NO: 1]
above, a packaging cell line or a helper adenovirus or
25   both may be necessary to provide sufficient adenovirus
gene sequences necessary for a productive viral infection
to generate the hybrid virus.

Helper viruses useful in this invention
contain selected adenovirus gene sequences not present in
30   the hybrid vector construct or expressed by the cell line
in which the hybrid vector is transfected. Optionally,
such a helper virus may contain a second reporter
minigene which enables s paration of th resulting hybrid
virus and the helper virus upon purification. The

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construction of desirable helper viruses is within the
skill of the art.

As one example, if the cell line employed
to produce the recombinant virus is not a packaging cell
5   line, and the hybrid vector contains only the minimum
adenovirus sequences identified above, the helper virus
may be a wild type Ad virus. Thus, the helper virus
supplies the necessary adenovirus early genes E1, E2a, E4
and all remaining late, intermediate, structural and non-
10   structural genes of the adenovirus genome. However, if,
in this situation, the packaging cell line is 293, which
supplies the E1 proteins, the helper virus need not
contain the E1 gene.

In another embodiment, when the hybrid
15   construct is rendered replication defective by a
functional deletion in E1 but contains no other deletions
in Ad genes necessary for production of an infective
viral particle, and the 293 cell line is employed, no
helper virus is necessary for production of the hybrid
20   virus. Additionally, all or a portion of the adenovirus
delayed early gene E3 (which spans mu 76.6 to 86.2) may
be eliminated from the helper virus useful in this
invention because this gene product is not necessary for
the formation of a functioning hybrid virus particle.
25   It should be noted that one of skill in
the art may design other helper viruses or develop other
packaging cell lines to complement the adenovirus
deletions in the vector construct and enable production
of the hybrid virus particle, given this information.
30   Therefore, this invention is not limited by the use or
description of any particular helper virus or packaging
c ll line.

Thus, as described in Figs. 1A through 1C,
the circular plasmid pAd.AV.CMVLacZ [SEQ ID NO: 1]
35   (containing the minigene and only adenovirus sequences

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from map unit 0 tc 1 and 9 to 16) was digested and co-
transfected with a selected Ad5 helper virus (containing
adenovirus sequences 9 to 78.4 and 86 to 100) into 293
cells. Thus, the packaging cell line provides the E1

5   proteins and the helper virus provides all necessary
adenovirus gene sequences subsequent to map unit 16.
Homologous recombination occurs between the helper virus
and the hybrid vector, resulting in the hybrid viral
particle. Growth of this hybrid viral particle in 293

10   cells has been closely monitored for greater than 20
rounds of amplification with no indication of genome
instability. Rescue and integration of the transgene
from the hybrid virus into a host cell and further
modifications of the vector are described below. The

15   resulting hybrid virus Ad.AV.CMV*LacZ* combines the high
titer potential of adenovirus with the integrating
biology associated with AAV latency.

G.   *Hybrid Virus Polycation Conjugates*

Rep expression is required for rescue of

20   the rAAV genome to occur. A preferred approach is to
synthetically incorporate a plasmid permitting expression
of *rep* into the hybrid particle. To do so, the hybrid
viruses described above are further modified by resort to
adenovirus-polylysine conjugate technology. See, e.g.,

25   Wu et al, *J. Biol. Chem.*, *264*:16985-16987 (1989); and K.
J. Fisher and J. M. Wilson, *Biochem. J.*, *299*: 49 (April
1, 1994), incorporated herein by reference. Using this
technology, a hybrid virus as described above is modified
by the addition of a poly-cation sequence distributed

30   around the capsid of the hybrid viral particle.
Preferably, the poly-cation is poly-lysine, which
attaches around th negatively-charged virus to form an
external positive charg . A plasmid containing the AAV
rep gene (or a functional portion th reof) under the

35   control of a suitable promoter is then complexed directly

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to the hybrid capsid, resulting in a single viral
particle containing the hybrid virus and an AAV *rep* gene.
The negatively charged plasmid DNA binds with high
affinity to the positively charged polylysine.

5   Essentially the techniques employed in constructing this
hybrid virus conjugate or trans-infection particle are as
described in detail in Example 3 below.

An alternative embodiment of the hybrid
vector and resulting viral particle is provided by

10   altering the *rep* containing plasmid to also contain an
AAV *cap* gene. This embodiment of the hybrid vector when
in a host cell is thus able to produce a recombinant AAV
particle, as discussed in more detail below.

The plasmids employed in these embodiments

15   contain conventional plasmid sequences, which place a
selected AAV sequence, i.e., *rep* and/or *cap* gene
sequences, under the control of a selected promoter. In
the example provided below, the exemplary plasmid is
pRep78/52 [SEQ ID NO: 2], a trans-acting plasmid

20   containing the AAV sequences that encode *rep* 78 kD and 52
kD proteins under the control of the AAV P5 promoter.
The plasmid also contains an SV40 polyadenylation signal.
The DNA sequence of this plasmid is provided in Fig. 8
[SEQ ID NO: 2].

25   In a similar manner and with resort to
plasmid and vector sequences known to the art, analogous
plasmids may be designed using both *rep* and *cap* genes,
and different constitutive or regulated promoters,
optional poly-A sequences and introns.

30   The availability of materials to make
these modified hybrid vectors and viruses and the AAV *rep*
and/or *cap* containing vectors and the techniques involved
in the assembly of th hybrid vector and *rep* and/ r *cap*
containing plasmids ar conventi nal as described above.

35   The assembly techniques for the trans-infection particle

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employ the techniques described above for the hybrid
vector and the techniques of Wu et al and Fisher et al,
cited above. The use of this trans-infection particle
including rescue and integration of the transgene into
5   the host cell is described below.

II.   Function of the Hybrid Virus
      A.   _The Hybrid Virus Infects a Target Cell_
            Once the hybrid virus or trans-infection
10   particle is constructed as discussed above, it is
targeted to, and taken up by, a selected target cell.
The selection of the target cell also depends upon the
use of the hybrid virus, i.e., whether or not the
transgene is to be replicated _in vitro_ for production of
15   a recombinant AAV particle, or _ex vivo_ for production
into a desired cell type for redelivery into a patient,
or _in vivo_ for delivery to a particular cell type or
tissue. Target cells may therefor be any mammalian cell
(preferably a human cell). For example, in _in vivo_ use,
20   the hybrid virus can target to any cell type normally
infected by adenovirus, depending upon the route of
administration, i.e., it can target, without limitation,
neurons, hepatocytes, epithelial cells and the like.
Uptake of the hybrid virus by the cell is caused by the
25   infective ability contributed to the vector by the
adenovirus and AAV sequences.
      B.   _The Transgene is Rescued._
            Once the hybrid virus or trans-infection
particle is taken up by a cell, the AAV ITR flanked
30   transgene must be rescued from the parental adenovirus
backbone. Rescue of the transgene is dependent upon
supplying the infected cell with an AAV _rep_ gene. Thus,
efficacy of the hybrid virus can be measured in terms of
rep mediated rescue f rAAV from the parental adenovirus
35   template.

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The *rep* genes can be supplied to the
hybrid virus by several methods. One embodiment for
providing *rep* proteins in *trans* was demonstrated with the
exemplary hybrid virus Ad.AV.CMV*LacZ* by transfecting into
5   the target monolayer of cells previously infected with
the hybrid vector, a liposome enveloped plasmid pRep78/52
[SEQ ID NO: 2] containing the genes encoding the AAV *rep*
78 kDa and 52 kDa proteins under the control of the AAV
P5 promoter. Rescue and amplification of a double-
10   stranded AAV monomer and a double-stranded AAV dimer,
each containing the *LacZ* transgene described above, was
observed in 293 cells. This is described in detail in
Example 2.

The production of *rep* in *trans* can be
15   modulated by the choice of promoter in the *rep* containing
plasmid. If high levels of *rep* expression are important
early for rescue of the recombinant AAV domain, a
heterologous (non-adenovirus, non-AAV) promoter may be
employed to drive expression of *rep* and eliminate the
20   need for E1 proteins. Alternatively, the low levels of
rep expression from P5 that occur in the absence of
adenovirus E1 proteins may be sufficient to initiate
rescue and optimal to drive integration of the
recombinant AAV genome in a selected use.

25   More preferably for *in vivo* use, the AAV
rep gene may also be delivered as part of the hybrid
virus. One embodiment of this single particle concept is
the polycation conjugated hybrid virus (see Fig. 7).
Infection of this trans-infection particle is
30   accomplished in the same manner and with regard to the
same target cells as identified above. The polylysine
conjugate of the hybrid virus onto which was directly
complexed a plasmid that encoded the *rep* 78 and 52
proteins, combines all of the functional components into
35   a single particle structure. Thus, th trans-infection

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particle permits delivery of a single particle to the
cell, which is considerably more desirable for
therapeutic use. Similar experiments to demonstrate
rescue of the transgene from the hybrid conjugate trans-
5   infection particle in 293 cells and in HeLa cells are
detailed in Example 4.

In another embodiment, the hybrid virus is
modified by cloning the *rep* cDNA directly into the
adenovirus genome portion of the hybrid vector. Because
10   it is known that even residual levels of *rep* expression
can interfere with replication of adenovirus DNA, such
incorporation of *rep* into the hybrid vector itself is
anticipated to requires possible mutation of the *rep*
genes to encode only selected domains, and the use of
15   inducible promoters to regulate *rep* expression, as well
as careful placement of the *rep* genes into the adenovirus
sequences of the hybrid vector.

C.   *Transgene Integrates into Chromosome*

Once uncoupled (rescued) from the genome
20   of the hybrid virus, the recombinant AAV/transgene
minigene seeks an integration site in the host chromatin
and becomes integrated therein, providing stable
expression of the accompanying transgene in the host
cell. This aspect of the function of the hybrid virus is
25   important for its use in gene therapy. The AAV/
transgene minigene sequence rescued from the hybrid virus
achieves provirus status in the target cell, i.e., the
final event in the hybrid lifecycle (Fig. 7).

To determine whether the AAV minigene
30   rescued from the hybrid virus achieves provirus status in
a target cell, non-E1 expressing HeLa cells were infected
with th hybrid vector-poly-Lysine conjugate complexed
with pR p78/52 [SEQ ID NO: 2] and passaged until stable
colonies of *LacZ* expressing cells are evident. A
35   duplicate plate of cells was inf cted with the sam

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conjugate, but instead of being complexed with the
pRep78/52 plasmid [SEQ ID NO: 2], carried an irrelevant
plasmid. Cells that receive the *rep* containing hybrid
particle produced a greater number of stable *LacZ*
5   positive colonies than cells injected with the control
vector. This indicates multiple rescue and integration
events in cells that expressed *rep* proteins.
Confirmation of integration is revealed by characterizing
the recombinant AAV genome in the hybrid infected cells
10 and identifying flanking chromosomal sequences (see
Example 5).

**III.   Use of the Hybrid Viruses and Viral Particles
in Gene Therapy**

15   The novel hybrid virus and trans-infection
particles of this invention provide efficient gene
transfer vehicles for somatic gene therapy. These hybrid
viruses are prepared to contain a therapeutic gene in
place of the *LacZ* reporter transgene illustrated in the
20 exemplary vector. By use of the hybrid viruses and
trans-infection particles containing therapeutic
transgenes, these transgenes can be delivered to a
patient *in vivo* or *ex vivo* to provide for integration of
the desired gene into a target cell. Thus, these hybrid
25 viruses and trans-infection particles can be employed to
correct genetic deficiencies or defects. Two examples of
the generation of gene transfer vehicles for the
treatment of cystic fibrosis and familial
hypercholesterolemia are described in Examples 6 and 7
30 below. One of skill in the art can generate any number
of other gene transfer vehicles by including a selected
transgene for the treatment of other disorders. For
example, the trans-infection particles are anticipated to
be particularly advantageous in *ex vivo* gene therapy

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where transduction and proviral integration in a stem
cell is desired, such as in bone marrow directed gene
therapy.

The hybrid viruses and trans-infection
5   particles of the present invention may be administered to
a patient, preferably suspended in a biologically
compatible solution or pharmaceutically acceptable
delivery vehicle. A suitable vehicle includes sterile
saline. Other aqueous and non-aqueous isotonic sterile
10 injection solutions and aqueous and non-aqueous sterile
suspensions known to be pharmaceutically acceptable
carriers and well known to those of skill in the art may
be employed for this purpose.

The hybrid viruses and trans-infection
15 particles of this invention may be administered in
sufficient amounts to transfect the desired cells and
provide sufficient levels of integration and expression
of the selected transgene to provide a therapeutic
benefit without undue adverse or with medically
20 acceptable physiological effects which can be determined
by those skilled in the medical arts. Conventional and
pharmaceutically acceptable routes of administration
include direct delivery to the target organ, tissue or
site, intranasal, intravenous, intramuscular,
25 subcutaneous, intradermal, oral and other parental routes
of administration. Routes of administration may be
combined, if desired.

Dosages of the hybrid virus and/or trans-
infection particle will depend primarily on factors such
30 as the condition being treated, the selected gene, the
age, weight and health of the patient, and may thus vary
among patients. A th rap utically effective human dos
of the hybrid viruses or trans-inf ction particles of the
present inv ntion is believ d to be in the range of from
35 about 20 to about 50 ml of saline solution containing

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concentrations of from about 1 x 10^7 to 1 x 10^{10} pfu/ml
hybrid virus of the present invention. A preferred human
dose is about 20 ml saline solution at the above
concentrations. The dosage will be adjusted to balance

5   the therapeutic benefit against any side effects. The
levels of expression of the selected gene can be
monitored to determine the selection, adjustment or
frequency of dosage administration.

10 **IV.   High Efficiency Production of rAAV**
The hybrid viruses and trans-infection
particles of this invention have another desirable
utility in the production of large quantities of
recombinant AAV particles. Due to the complicated

15 current methods for generating AAV, there is only a
limited amount of AAV available for use in industrial,
medical and academic biotechnology procedures. The
vectors and viruses of the present invention provide a
convenient and efficient method for generating large

20 quantities of rAAV particles.
According to this aspect of the invention, a
trans-infection particle is constructed as described
above and in Example 3 and is employed to produce high
levels of rAAV as detailed in Example 8, with the

25 possible modifications described in Example 9 below.
Briefly, a plasmid is generated that contains both AAV
rep and *cap* genes under the control of a suitable plasmid
and is complexed to the poly-lysine exterior of the
hybrid virus as described above. This trans-infection

30 particle is then permitted to infect a selected host
cell, such as 293 cells. The presence of both *rep* and
cap permit th formati n of AAV particles in the cells
and generat an AAV virus titer of about 10^9 virions. In
contrast, current meth ds involving the transfection of

35 multiple plasmids produce only about 10^7 virion tit r.

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The rAAV is isolated from the culture by selecting the
LacZ-containing blue plaques and purifying them on a
cesium chloride gradient.

5   The benefit of this procedure relates to the
fact that the cis AAV element is encoded by the parental
adenovirus genome. As a result, the trans plasmid is the
only DNA component that is needed for complex formation.
The cell is thereby loaded with significantly more copies
of the trans-acting rep and cap sequences, resulting in
10 improved efficiency of rescue and packaging.

Numerous comparative studies focusing on the
optimal ratio and copy number of the cis and trans
plasmids for AAV production indicated that there is a
positive correlation between the trans plasmid copy
15 number and yield of recombinant virus. As described in
detail in Example 8, the yield of recombinant AV.CMVLacZ
virus was increased by 5-10 fold by using the trans-
infection particle instead of a standard adenovirus
vector.

20   The primary limitation associated with the
production of recombinant AAV using a hybrid virus of
this invention relates to difficulties that arise in
distinguishing between the two viruses (i.e., adenovirus
and AAV) that are produced by the cell. Using the
25 exemplary vectors and vector components of this
invention, LacZ histochemical staining could not be used
to titer the yield of recombinant AV.CMVLacZ since any
contaminating Ad.AV.CMVLacZ hybrid would contribute to
the final count. Therefore, a rapid Southern blot
30 technique for quantitating yields of recombinant AAV was
incorporated. The assay that was developed enabled not
only quantitation and verificati n of AAV production, but
also demonstrated the r moval f contaminating hybrid
virus from purified AAV stocks.

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Another method for detecting contaminating
hybrid virions involves modifying the hybrid vector by
inserting a small second reporter minigene (i.e.,
reporter gene, promoter and other expression control
5   sequences, where desired) into the E3 region of the
parental adenovirus backbone. Because this reporter is
not linked to the AAV domain, contaminating hybrid virus
that is present during purification can be monitored by
this hybrid-specific marker. Another possible reporter
10   gene is the nucleic acid sequence for green fluorescent
protein. With this hybrid vector containing two reporter
sequences, histochemical staining for alkaline
phosphatase (adenovirus reporter) or β-galactosidase (AAV
reporter) activity can be used to monitor each viral
15   domain.

The following examples illustrate the
construction and testing of the hybrid vectors of the
present invention and the use thereof in the productions
of recombinant AAV. These examples are illustrative
20   only, and do not limit the scope of the present
invention.

Example 1 - Construction of a Hybrid Virus

A first hybrid adenovirus-AAV virus was
25   engineered by homologous recombination between DNA
extracted from an adenovirus and a complementing vector
according to protocols previously described [see, e.g.,
K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702
(1994) and references cited therein]. The following
30   description refers to the diagram of Fig. 1.

Adenovirus DNA was extracted from CsCl purified
d17001 virions, an Ad5 (serotype subgroup C) variant that
carries a 3 kb deletion between mu 78.4 through 86 in the
nonessential E3 r gion (provided by Dr. William Wold,
35   Washington University, St. Louis, Missouri). Adenoviral

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DNA was prepared for co-transfection by digestion with
ClaI (adenovirus genomic bp position 917) which removes
the left arm of the genome encompassing adenovirus map
units 0-2.5. See lower diagram of Fig. 1B.

5   The complementing hybrid vector, pAd.AV.CMVLacZ
(see Fig. 1A and Fig. 2 [SEQ ID NO: 1]) was constructed
as follows:

           A parental cloning vector, pAd.BglII was
designed. It contains two segments of wild-type Ad5
10   genome (i.e., map units 0-1 and 9-16.1) separated by a
unique BglII cloning site for insertion of heterologous
sequences. The missing Ad5 sequences between the two
domains (adenovirus genome bp 361-3327) results in the
deletion of E1a and the majority of E1b following
15   recombination with viral DNA.

           A recombinant AAV genome (AV.CMVLacZ) was
designed and inserted into the BglII site of pAd.BglII to
generate the complementing plasmid. The linear
arrangement of AV.CMVLacZ [SEQ ID NO: 1] (see top diagram
20   of Fig. 1B) includes:

           (a) the 5' AAV ITR (bp 1-173) obtained by PCR
using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)]
as template [nucleotide numbers 365-538 of Fig. 2 [SEQ ID
NO: 1]];

25   (b) a CMV immediate early enhancer/promoter
[Boshart et al, Cell, 41:521-530 (1985); nucleotide
numbers 563-1157 of Fig. 2 [SEQ ID NO: 1]],

           (c) an SV40 splice donor-splice acceptor
(nucleotide numbers 1178-1179 of Fig. 2 [SEQ ID NO: 1]),

30   (d) E. coli beta-galactosidase cDNA
(nucleotide numbers 1356 - 4827 of Fig. 2 [SEQ ID NO:
1]),

           (e) an SV40 polyadenylation signal (a 237 Bam
HI-BclI restriction fragment containing the
35   cleavage/poly-A signals from both the early and late

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transcription units; nucleotide numbers 4839 - 5037 of
Fig. 2 [SEQ ID NO: 1]) and

                (f)   3'AAV ITR, obtained from pAV2 as a SnaBI-
BglII fragment (nucleotide numbers 5053 - 5221 of Fig. 2
5   [SEQ ID NO: 1]).

                The resulting complementing hybrid vector,
pAd.AV.CMVLacZ (see Fig. 1A and Fig. 2 [SEQ ID NO: 1]),
contained a single copy of recombinant AV.CMVLacZ flanked
by adenovirus coordinates 0-1 on one side and 9-16.1 on

10   the other. Plasmid DNA was linearized using a unique
NheI site immediately 5' to adenovirus map unit zero (0)
(resulting in the top diagram of Fig. 1B).

                Both the adenovirus substrate and the
complementing vector DNAs were transfected to 293 cells

15   [ATCC CRL1573] using a standard calcium phosphate
transfection procedure [see, e.g., Sambrook et al, cited
above]. The end result of homologous recombination
involving sequences that map to adenovirus map units 9-
16.1 is hybrid Ad.AV.CMVLacZ (see Fig. 1C) in which the

20   E1a and E1b coding regions from the d17001 adenovirus
substrate are replaced with the AV.CMVLacZ from the
hybrid vector.

                Twenty-four hours later, the transfection
cocktail was removed and the cells overlayed with 0.8%

25   agarose containing 1x BME and 2% fetal bovine serum
(FBS). Once viral plaques developed (typically 10-12
days post-transfection), plaques were initially screened
for *E. coli* β-galactosidase (*LacZ*) activity by overlaying
the infected monolayer with agarose supplemented with a

30   histochemical stain for *LacZ*, according to the procedure
described in J. Price et al, Proc. Natl. Acad. Sci., USA,
84:156-160 (1987). Positive cl nes (identified by the
deposit of insoluble blue dye) were isolated, subjected
to three rounds of fre ze (dry ice/ethanol)- thaw (37°C)

35   and an aliquot f the suspended plaque was used to infect

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a fresh monolayer of 293 cells seeded on duplicate 60mm plates.

Twenty-four hours later the cells from one set of plates were fixed and again stained for *LacZ* activity.

5   Cells from the duplicate plate w₋re harvested, suspended in 0.5 ml 10 mM Tris-Cl, pH8.0, and lysed by performing a series of three freeze (dry ice/ethanol)-thaw (37°C) cycles. Cell debris was removed by centrifugation and an aliquot of the supernatant used to measure *LacZ* enzyme

10 activity.

As indicated in Fig. 3, assays for β-galactosidase activity which measured the absorbance at 420 nm of the beta-galactosidase blue color in successful recombinants, revealed that eight of the ten isolated,

15 putative positive clones (D1A through D1J) expressed high levels of enzyme. Histochemical staining produced similar results.

Large-scale production and purification of recombinant virus was performed as described in Kozarsky

20 et al, cited above, and references cited therein.

Example 2 - Functional Analysis of Hybrid Vector

The ability to rescue the AV.CMV*LacZ* sequence [SEQ ID NO: 1] from the hybrid virus represented an

25 important feature of the hybrid vector and virus systems of Example 1. To evaluate this feature, it was necessary to provide the necessary AAV gene products in *trans* that direct AAV excision and amplification (i.e. *rep* proteins). Furthermore, this experiment was conducted in

30 293 cells to transcomplement the E1 deletion in the Ad.AV.CMV*LacZ* clones, because the adenovirus E1 gene proteins have been shown to be important for initiating the lytic phase of the AAV lif cycle.

293 cells were seeded onto 6-w 11 35 mm plates

35 at a density of 1×10^6 cells/well. Twenty-four hours

later, seeding media [DMEM/10% FBS supplemented with antibiotics] was replaced with 1.0 ml DMEM/2% FBS and infected with Ad.AV.CMVLacZ hybrid clones at an MOI of 1. Two hours later, each well was transfected with 1 μg

5 plasmid pRep78/52 [SEQ ID NO: 2], a trans-acting plasmid that encodes the sequence encoding the AAV *rep* 78 kD and 52 kD proteins. The *rep* sequences in this construct are under the control of the AAV P5 promoter and utilize an SV40 polyadenylation signal.

10 As a positive control for AAV rescue, 293 cells seeded in a 6-well plate as above were co-transfected with a cis-acting AAV plasmid pAV.CMVLacZ and pRep78/52. pAV.CMVLacZ contained AV.CMVLacZ, the identical sequence encoded by pAd.AV.CMVLacZ [SEQ ID NO: 1] described in

15 Example 1 cloned into the BglII site of pSP72 (Promega).

To provide the necessary adenovirus helper function for AAV rescue, cells were infected with either wild-type Ad5 virus or a first generation E1-deleted virus Ad.CMhpAP at an MOI of 5, approximately 2 hours

20 prior to adding the transfection cocktail. Ad.CMhpAP is identical to Ad.CMVLacZ (Example 1) with the modification that the alkaline phosphatase sequence (which can be obtained from Genbank) is inserted in place of the LacZ gene.

25 Transfections were performed with Lipofectamine (Life Technologies) according to the instructions provided by the manufacturer. Thirty hours post-transfection, the cells were harvested and episomal DNA (Hirt extract) prepared as described by J. M. Wilson et

30 al, J. Biol. Chem., 267:(16):11483-11489 (1992). Samples were resolved on a 1.2% agarose gel and electroblotted onto a nylon membrane. Blots w re hybridized (Southern) with a ^{32}P random prim r-label d r striction fragment isolated from the *E. coli* LacZ cDNA.

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The full spectrum of duplex molecular species
that appear during a lytic AAV infection (i.e., monomeric
forms of the double stranded intermediates, RFm and RFd,
respectively) were evident in transfected cells infected
5   with wild type and El deleted Ad.. No replicative
intermediates were detected when transfections were
performed in the absence of helper virus.

Hirt extracts from the 293 cells infected with
putative Ad.AV.CMVLacZ hybrid clones D1A and D1C revealed
10   a single band corresponding to the viral DNA, when probed
with a LacZ restriction fragment. In the presence of rep
proteins 78 and 52, however, the same clones yielded a
banding pattern that included not only the adenovirus
DNA, but an RF monomer and dimer of AV.CMVLacZ. A
15   single-stranded form of AV.CMVLacZ [SEQ ID NO: 1] was not
evident. Two additional clones gave similar banding
patterns, D1B and D1H. In all, each of the eight
Ad.AV.CMVLacZ hybrids that were found in Fig. 3 to
express high levels of Lac Z activity were positive for
20   rescue of the AAV domain.

With the exception of an extra band of
approximately 3.5 kb, the rescue of the AV.CMVLacZ [SEQ
ID NO: 1] from the hybrid viral DNA was nearly identical
to results obtained from a standard cis and trans
25   plasmid-based approach. In these later samples,
adenovirus helper function was provided by pre-infecting
cells with either wild-type Ad5 or an El-deleted
recombinant virus Ad.CBhpAP (also termed H5.CBALP). The
Ad.CBhpAP virus has the same sequence as the Ad.CMhpAP
30   virus described above, except that the CMV promoter
sequence is replaced by the chicken cytoplasmic ß-actin
promoter [nucleotides +1 to +275 as described in T. A.
Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. The
level of rescue in c lls infected with WT Ad5 appeared to
35   be greater relative to those infected with the

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recombinant Ad.CBhpAP virus, likely due to the additional
E1 expression provided by the wild-type genome. The
relevance of including an E1 deleted adenovirus here is
to document that the level of adenovirus E1 proteins
5   expressed in 293 cells is sufficient for AAV helper
function.

Example 3 - Synthesis of Polylysine Conjugates
               Another version of the viral particle of this
10   invention is a polylysine conjugate with a *rep* plasmid
complexed directly to the hybrid virus capsid. This
conjugate permits efficient delivery of the *rep*
expression plasmid pRep78/52 [SEQ ID NO: 2] in tandem
with the hybrid virus, thereby removing the need for a
15   separate transfection step. See, Fig. 8 for a
diagrammatic outline of this construction.
               Purified stocks of a large-scale expansion of
Ad.AV.CMVLacZ clone D1A were modified by coupling poly-L-
lysine to the virion capsid essentially as described by
20   K. J. Fisher and J. M. Wilson, Biochem. J., 299:49-58
(1994), resulting in an Ad.AV.CMVLacZ-$(Lys)_n$ conjugate.
The procedure involves three steps. First, hybrid
virions are activated through primary amines on capsid
proteins with the heterobifunctional water-soluble cross-
25   linking agent, sulpho-SMCC [sulpho-(N-succinimidyl 4-(N-
maleimidomethyl)-cyclohexane-1-carboxylate] (Pierce).
The conjugation reaction, which contained 0.5 mg (375
nmol) of sulpho-SMCC and 6 x 10^{12} A_{260} hybrid vector
particles in 3.0 ml of HBS, was incubated at 30°C for 45
30   minutes with constant gentle shaking. This step involved
formation of a peptide bond between the active N-
hydroxysuccinimide (NHS) ester of sulpho-SMCC and a free
amine (e.g. lysine) contributed by an adenovirus protein
sequence (capsid prot in) in th recombinant virus,
35   yielding a maleimide-activated viral particle.

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Unincorporated, unreacted cross-linker was removed by gel filtration on a 1 cm x 15 cm Bio-Gel P-6DG (Bio-Rad Laboratories) column equilibrated with 50 mM Tris/HCl buffer, pH 7.0, and 150 mM NaCl. Peak A_{260}

5 fractions containing maleimide-activated hybrid virus were combined and placed on ice.

Second, poly-L-lysine having a molecular mass of 58 kDa at 10 mg/ml in 50 mM triethanolamine buffer (pH 8.0), 150 mM NaCl and 1 mM EDTA was thiolated with 2-

10 imminothiolane/HCl (Traut's Reagent; Pierce) to a molar ratio of 2 moles-SH/mole polylysine under N_2; the cyclic thioimidate reacts with the poly(L-lysine) primary amines resulting in a thiolated polycation. After a 45 minute incubation at room temperature the reaction was applied

15 to a 1 cm x 15 cm Bio-Gel P6DG column equilibrated with 50 mM Tris/HCl buffer (pH 7.0), 150 mM NaCl and 2 mM EDTA to remove unincorporated Traut's Reagent.

Quantification of free thiol groups was accomplished with Ellman's reagent [5,5'-dithio-bis-(2-

20 nitrobenzoic acid)], revealing approximately 2 mol of -SH/mol of poly(L-lysine). The coupling reaction was initiated by adding 1×10^{12} A_{260} particles of maleimide-activated hybrid virus/mg of thiolated poly(L-lysine) and incubating the mixture on ice at 4°C for 15 hours under

25 argon. 2-mercaptoethylamine was added at the completion of the reaction and incubation carried out at room temperature for 20 minutes to block unreacted maleimide sites.

Virus-polylysine conjugates, Ad.AV.CMVLacZ-

30 (Lys)$_n$, were purified away from unconjugated poly(L-lysine) by ultracentrifugation through a CsCl step gradient with an initial composition of equal volumes of 1.45 g/ml (bottom step) and 1.2 g/ml (top step) CsCl in 10 mM Tris/HCl buffer (pH 8.0). Centrifugation was at

35 90,000 g for 2 hours at 5°C. The final product was

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dialyzed against 20 mM Hepes buffer (pH 7.8) containing
150 mM NaCl (HBS).

Complexes of Ad.AV.CMVLacZ-(Lys)$_n$ with
pRep78/52 plasmid DNA [SEQ ID NO: 2] were formed by
5 adding varying quantities of Ad.AV.CMVLacZ-(Lys)$_n$ in 50μl
HBS to 0.5 μg of pRep78/52 plasmid DNA [SEQ ID NO: 2] in
50μl HBS. After 30 minutes incubation at room
temperature, a complex was formed of the hybrid virus
Ad.AV.CMVLacZ-(Lys)$_n$ associated in a single particle with
10 the plasmid DNA containing the rep genes.

This complex, termed a trans-infection
particle, was evaluated for DNA binding capacity by gel
mobility shift assays performed as described in Fisher et
al, cited above. This analysis revealed that the plasmid
15 binding capacity of the purified conjugate (expressed as
the number of A$_{260}$ particles Ad.AV.CMVLacZ-(Lys)$_n$ that
can neutralize the charge contributed by 1 μg plasmid
DNA) was 1 μg pRep78/52 plasmid DNA/6.0 x 10^{10} A$_{260}$
particles Ad.AV.CMVLacZ-(Lys)$_n$.

20

Example 4 - Trans-Infection Protocol to Demonstrate AAV
Excision and Amplification

Trans-infection complexes were prepared by
mixing Ad.AV.CMVLacZ-(Lys)$_n$ conjugate with pRep78/52
25 plasmid [SEQ ID NO: 2] and applied to 293 cells as
follows. Ad.AV.CMVLacZ-(Lys)$_n$ (6 x 10^{10} A$_{260}$ particles) in
100 μl DMEM was added dropwise to a microfuge tube
containing 1 μg plasmid DNA in 100 μl DMEM. The mixture
was gently mixed and allowed to incubate at room
30 temperature for 10-15 minutes. The trans-infection
cocktail was added to 293 cells seeded in a 35 mm 6-well
as detailed above. Thirty hours later, cells were
harvested and Hirt extracts prepared.

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Samples were resolved on a 1.2% agarose gel and
electroblotted onto a nylon membrane. Blots were
hybridized (Southern) with a P-32 random primer-labeled
restriction fragment isolated from the *E. coli LacZ* cDNA.

5   The Hirt extracts from 293 cells revealed a
banding pattern that suggested the AV.CMV*LacZ* minigene
sequence [SEQ ID NO: 1] was efficiently rescued from the
hybrid conjugate. Both an RF monomer and dimer of the
recombinant AV.CMV*LacZ* sequence were evident. As was

10   observed previously, the rescue event was dependent on
rep proteins since 293 cells that were trans-infected
with a hybrid conjugate complexed with an irrelevant
reporter plasmid expressing alkaline phosphatase (i.e.
pCMVhpAP) revealed only Ad.AV.CMV*LacZ* DNA. This negative

15   control for rescue was secondarily useful for
demonstrating the high efficiency of gene transfer to 293
cells that was achieved with the conjugate vehicle.

         A duplicate set of 293 cells that received
hybrid conjugate which was further complexed with

20   alkaline phosphatase expression plasmid were fixed 24
hours after addition of the trans-infection cocktail and
histochemically stained for *LacZ* as described in Price et
al, cited above, or for alkaline phosphatase activity as
described in J. H. Schreiber et al, BioTechniques,

25   14:818-823 (1993). Here *LacZ* was a marker for the
Ad.AV.CMV*LacZ* hybrid, while alkaline phosphatase served
as a reporter for the carrier plasmid. Greater than 90%
of the monolayer was transduced with both β-galactosidase
and alkaline phosphatase transgenes, showing the high

30   efficiency of the conjugate delivery vehicle
(differential staining revealed a blue color for the
hybrids containing the *LacZ* marker and a purple color for
the plasmids bearing th AP marker).

         Because of the important role E1 proteins have

35   for progression of the AAV lifecycle, it was critical to

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test the efficiency of the hybrid delivery system in a
setting where E1 proteins are not expressed. A
trans-infection experiment using the hybrid conjugate
complexed with pRep78/52 [SEQ ID NO: 2] was therefore
5   conducted in HeLa cells [ATCC CCL2] to remove the
involvement of E1 proteins. The findings suggested
rescue of AV.CMVLacZ occurred evidenced by the
accumulation of RF monomers and dimers. Rescue from HeLa
cells (which unlike the 293 cells do not contain any
10   adenovirus E1 proteins) revealed lower levels of rescue
of the transgene. The expression of *rep* from the AAV P5
promoter is upregulated by adenovirus E1 and signals the
beginning of the AAV lytic cycle. In the absence of E1,
rep expression from the P5 promoter is virtually silent
15   which is important for maintenance of the proviral latent
stages of the AAV lifecycle. It is anticipated that a
promoter not dependent on E1 expression will upon
substitution for P5, overcome this problem.

20   <u>Example 5 - Integration of the Transgene</u>
                A preliminary study has been performed to
determine whether the AAV sequence rescued from the
hybrid virus can achieve provirus status in a target cell
(Fig. 7). Briefly, HeLa cells [ATCC CCL 2] were infected
25   with the hybrid conjugate complexed with pRep78/52 [SEQ
ID NO: 2] and passaged until stable colonies of *LacZ*
expressing cells were evident. A duplicate plate of
cells was infected with the same conjugate, but instead
of being complexed with the pRep78/52 plasmid [SEQ ID NO:
30   2], carried an irrelevant plasmid. These findings
indicated that cells that received the Rep containing
hybrid particle produced a greater number of stable *LacZ*
positiv colonies than cells that were infected with the
control virus. This could be interpreted as a reflection
35   of multiple rescue and integrati n events in cells that

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expressed Rep proteins. However, it is possible that an
episomal form of AAV that can persist for extended
periods of time was present.

5   To establish the occurrence of integration into
the chromosome of the minigene Av.CMVLacZ from the hybrid
conjugate, the following experiment is performed. The
Ad.AV.CMVLacZ-(Lys)$_n$ conjugate carrying pRep78/52 plasmid
[SEQ ID NO: 2] is used to infect HeLa cells [ATCC CRL2]
(primary fibroblasts may also be used). The infected
10   cells are passaged for several generations. The cells
are grown to confluency, split and allowed to grow to
confluency again, split again and this cycle repeated as
desired. This permits sufficient time for uptake,
expression, replication and integration to occur. See
15   Fig. 7.

To verify that the recombinant AAV sequence
that was rescued from the hybrid genome (step III of Fig.
7) has integrated into a chromosome of the host cell
(step IV of Fig. 7), cells are separated by a
20   Fluorescence Activated Cell Sorter (FACS). By this
technique, those cells containing a stable integrated
copy of the recombinant AV.CMVLacZ minigene are separated
based on the presence of the β-galactosidase reporter.
These cells are tagged with fluorescein-labeled
25   antibodies that recognize the β-Gal protein, and are then
separated from non-transduced cells (i.e. those that did
not receive a copy of the AAV minigene) by FACS.

DNA is isolated from this purified population
of cells and used to construct a genomic library which is
30   screened for individual clones and the sequence verified.
If integration occurs, it is documented directly by
sequence analysis.

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Example 6 - Gene Transfer Vehicle for Cystic Fibrosis

An adenovirus-AAV-CFTR virus constructed by
modifying the hybrid Ad.AV.CMV*lacZ* virus described in
Example 1 to contain the cystic fibrosis transmembrane
5   regulator (CFTR) gene [J.R. Rioidan et al, Science,
245:1066-1073 (1989)] in place of the lacZ gene, using
known techniques. One suitable method involves producing
a new vector using the techniques described in Example 1.
In this new vector the *LacZ* minigene is replaced with the
10   CFTR minigene. For performance of this method vectors
bearing the CFTR gene have been previously described and
can be readily constructed. This new or reconstructed
vector is used to generate a new virus through homologous
recombination as described above. The resulting hybrid
15   virus is termed hybrid Ad.AV.CMV*CFTR*. It has the
sequence of Fig. 2 [SEQ ID NO: 1], except that the *LacZ*
gene is replaced with CFTR. Alternatively, the *LacZ* gene
can be removed from the Ad.AV.CMV*LacZ* vector of Example 1
and replaced with the CFTR gene using known techniques.
20   This virus (or an analogous hybrid virus with a
different promoter, regulatory regions, etc.) is useful
in gene therapy alone, or preferably, in the form of a
conjugate prepared as described in Example 4.

Treatment of cystic fibrosis, utilizing the
25   viruses provided above, is particularly suited for *in
vivo*, lung-directed, gene therapy. Airway epithelial
cells are the most desirable targets for gene transfer
because the pulmonary complications of CF are usually its
most morbid and life-limiting. Thus, the hybrid vector
30   of the invention, containing the CFTR gene, is delivered
directly into the airway, e.g. by formulating the hybrid
virus above, into a preparation which can be inhaled.
For xample, the hybrid virus or c njugate of the
inv ntion containing th CFTR gen , is suspended in 0.25

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molar sodium chloride. The virus or conjugate is taken
up by respiratory airway cells and the gene is expressed.

Alternatively, the hybrid viruses or conjugates
of the invention may be delivered by other suitable
5   means, including site-directed injection of the virus
bearing the CFTR gene. In the case of CFTR gene
delivery, preferred solutions for bronchial instillation
are sterile saline solutions containing in the range of
from about 1×10^7 to 1×10^{10} pfu/ml, more particularly,
10   in the range of from about 1×10^8 to 1×10^9 pfu/ml of
the recombinant hybrid virus of the present invention.

Other suitable methods for the treatment of
cystic fibrosis by use of gene therapy recombinant
viruses of this invention may be obtained from the art
15   discussions of other types of gene therapy vehicles for
CF. See, for example, U. S. Patent No. 5,240,846,
incorporated by reference herein.

Example 7 - Gene Transfer Vehicle for Familial
20   Hypercholesterolemia

Familial hypercholesterolemia (FH) is an
autosomal dominant disorder caused by abnormalities
(deficiencies) in the function or expression of LDL
receptors [M.S. Brown and J.L. Goldstein, Science,
25   232(4746):34-37 (1986); J.L. Goldstein and M.S. Brown,
"Familial hypercholesterolemia" in Metabolic Basis of
Inherited Disease., ed. C.R. Scriver et al, McGraw Hill,
New York, pp1215-1250 (1989).] Patients who inherit one
abnormal allele have moderate elevations in plasma LDL
30   and suffer premature life-threatening coronary artery
disease (CAD). Homozygous patients have severe
hypercholesterolemia and life-threatening CAD in
childhood.

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A hybrid adenovirus-AAV-LDL virus of the
invention is constructed by replacing the lacZ gene in
the hybrid Ad.AV.CMVlacZ virus of Example 1 with an LDL
receptor gene [T. Yamamoto et al, _Cell_, 39:27-38 (1984)]

5   using known techniques and as described analogously for
CF in the preceding example. Vectors bearing the LDL
receptor gene can be readily constructed according to
this invention. The resulting hybrid vector is termed
pAd.AV.CMVLDL.

10 This plasmid or its recombinant virus is useful
in gene therapy of FH alone, or preferably, in the form
of a viral conjugate prepared as described in Example 4
to substitute a normal LDL gene for the abnormal allele
responsible for the gene.

15 A.   Ex Vivo Gene Therapy
Ex vivo gene therapy can be performed by
harvesting and establishing a primary culture of
hepatocytes from a patient. Known techniques may be used
to isolate and transduce the hepatocytes with the above

20 vector(s) bearing the LDL receptor gene(s). For example,
techniques of collagenase perfusion developed for rabbit
liver can be adapted for human tissue and used in
transduction. Following transduction, the hepatocytes
are removed from the tissue culture plates and reinfused

25 into the patient using known techniques, e.g. via a
catheter placed into the inferior mesenteric vein.

B.   In Vivo Gene Therapy
Desirably, the _in vivo_ approach to gene
therapy, e.g. liver-directed, involves the use of the

30 hybrid viruses and viral conjugates described above. A
preferred treatment involves infusing a trans-infection
particle of the invention containing LDL into the
peripheral circulation of the patient. The patient is
then evaluated for change in serum lipids and liver

35 tissues.

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The hybrid virus or viral conjugate can be
used to infect hepatocytes *in vivo* by direct injection
into a peripheral or portal vein (10^7-10^8 pfu/kg) or
retrograde into the biliary tract (same dose). This
5   effects gene transfer into the majority of hepatocytes.
Treatments are repeated as necessary, e.g.
weekly. Administration of a dose of virus equivalent to
an MOI of approximately 20 (i.e. 20 pfu/hepatocyte) is
anticipated to lead to high level gene expression in the
10   majority of hepatocytes.

Example 8 - Efficient Production of Recombinant AAV using
A Hybrid Virus/Conjugate
The following experiment demonstrated that the
15   AAV genome that was rescued from the Ad.AV.CMV*LacZ* hybrid
virus could be packaged into an AAV capsid, provided the
cap open reading frame was supplied in trans. Thus the
viruses of this invention are useful in a production
method for recombinant AAV which overcomes the prior art
20   complications that surround the high titer production of
recombinant AAV.
A.   Trans-Infection Protocol for the
Production of rAAV
A trans-infection complex was formed
25   composed of the Ad.AV.CMV*LacZ*-(Lys)$_n$ conjugate described
above and a transcomplementing plasmid pAdAAV, which is
described in detail in R. J. Samulski et al, J. Virol.,
63(9):3822-3828 (1989)]. Briefly, plasmid pAdAAV encodes
the entire *rep* and *cap* open reading frames in the absence
30   of AAV ITRs, and has been shown to provide the necessary
AAV helper functions for replication and packaging of
recombinant AAV sequences.
Ad.AV.CMV*LacZ*-(Lys)$_n$ conjugate (4.5 x 10^{13}
A$_{260}$ particles) in 75 ml DMEM was added dropwise with
35   constant g ntle swirling in 25 ml DMEM containing 750 μg

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pAdAAV helper plasmid and incubated at room temperature
for 10-15 minutes. The complex was diluted with 450 ml
DMEM supplemented with 2% FBS and 20 ml aliquots were
added to monolayers of 293 cells seeded on 150 mm plates.

5   Forty hours post trans-infection, cells were
harvested, suspended in 12 ml 10 mM Tris-Cl (pH 8.0), and
stored at -80°C.

        Because the anticipated outcome was the
production of hybrid virus Ad.AV.CMVLacZ and a

10   recombinant AAV virion (AV.CMVLacZ), both of which carry
a functional LacZ minigene, it was not possible to use
detection of LacZ activity as an indicator of AV.CMVLacZ
production. A novel molecular approach was developed
that could be performed in one day and permitted

15   identification of the packaged viral DNAs.

        B.   Purification of rAAV

        Briefly, frozen cell suspensions were
subjected to three rounds of freeze-thaw cycles to
release recombinant AV.CMVLacZ and hybrid Ad.AV.CMVLacZ.

20   On completion of the final thaw, bovine pancreatic DNAse
(2000 units) and ribonuclease (0.2 mg/ml final
concentration) was added and the extract incubated at
37°C for 30 minutes. Cell debris was removed by
centrifugation (5000xg for 10 minutes) and the clarified

25   supernatant (15 ml) applied to a 22.5 ml step gradient
composed of equal volumes of CsCl at 1.2 g/ml, 1.36 g/ml,
and 1.45 g/ml 10mM Tris-Cl, pH8.0. Viral particles were
banded at 25,000 rpm in a Beckman SW-28 rotor for 8 hours
at 4°C. One ml fractions were collected from the bottom

30   of the tube.

        The fractions retrieved from the CsCl
gradient of partially purified virus are then digested to
release viral DNA from virion capsids as follows. A
5.0μl sample of each fraction was transferred to a

35   microfuge tube containing 20 μl capsid digestion buffer

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(50mM Tris-Cl, pHC.0, 1.0mM EDTA, pH8,0, 0.5% SDS, and
1.0 mg/ml Proteinase K). The reaction was incubated at
50°C for 1 hour, allowed to cool to room temperature,
diluted with 10 μl milli-Q water, and agarose gel loading
5   dye added.

These fractions are then analyzed by
Southern blotting. Samples were resolved on a 1.2%
agarose gel, electroblotted onto a nylon membrane. A ^{32}P
labeled *LacZ* restriction fragment which was common to
10   both vectors was used as a hybridization probe to locate
the migration of viral DNA through the agarose gel.
Viral bands were quantitated on a Molecular Dynamics
Phosphoimager.

A sample of the extract before CsCl
15   banding was also tested and revealed both hybrid
Ad.AV.CMV*LacZ* DNA and double-stranded RF forms (monomers
and dimers) of the rescued AV.CMV*LacZ* sequence [SEQ ID
NO: 1]. A single-stranded monomer of AV.CMV*LacZ* appeared
to be present in the crude extract; however, it was not
20   until the virions were concentrated by buoyant density
ultracentrifugation that the single-stranded genome
became clearly evident. The single-stranded recombinant
genome of the virus was distributed over a range of CsCl
densities and revealed a biphasic banding pattern. The
25   two peaks of single-stranded rAAV genome occurred at
densities of 1.41 and 1.45 g/ml CsCl, consistent with the
reported buoyant densities of wild-type AAV in CsCl [L.
M. de la Maza et al, *J. Virol.*, 33:1129-1137 (1980)].
Analysis of the fractions corresponding to the two vector
30   forms revealed the rAAV-1.41 species was several orders
of magnitude more active for *lacZ* transduction than the
denser rAAV-1.45 g/ml variant. To avoid confusion with
contaminating Ad.AAV, samples were heat inactivated (60°C
for 30 min) before being added to indicator HeLa cells.

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The peak fractions of rAAV-1.41 were combined and purified by equilibrium sedimentation in CsCl to eliminate residual adenovirus particles and concentrate rAAV virions. On the final round of

5 ultracentrifugation, a faint but clearly visible opalescent band was observed in the middle of the gradient tube. Fractions that surrounded the band were evaluated for density, absorbance at 260 nm, and lacZ transducing particles. As the band eluted from the

10 gradient tube, a well defined peak of 260 nm absorbing material was recorded, with a maximal absorbance occurring at a density of 1.40 g/ml CsCl. Analysis for lacZ transducing particles on HeLa cells revealed a peak of activity that mirrored the absorbance profile. These

15 results indicate rAAV was produced from the hybrid Ad.AAV virus. Furthermore, the titers achieved using the hybrid virus were 5-10 fold elevated compared to more conventional recombinant AAV production schemes (i.e., transfections with cis- and trans-acting plasmids). This

20 represents a significant improvement in rAAV production and indicates that the hybrid is useful for large-scale rAAV production.

All references recited above are incorporated herein by reference. Numerous modifications and

25 variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as those modifications permitting

30 optimal use of the hybrid viruses as gene therapy vehicles or production vehicles for recombinant AAV production, are believed to be encompass d in the scope of the claims appended hereto.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: Trustees of the University of Pennsylvania
               Wilson, James M.
               Kelley, William M.
               Fisher, Krishna J.

   (ii) TITLE OF INVENTION: Hybrid Adenovirus-AAV Vector and
                          Methods of Use Thereof

  (iii) NUMBER OF SEQUENCES: 2

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    (v) COMPUTER READABLE FORM:
       (A) MEDIUM TYPE: Floppy disk
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       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10398 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT          50

ATGATAATGA GGGGGTGGAG TTTGTGACGT GGCGCGGGGC GTGGGAACGG         100

GGCGGGTGAC GTAGTAGTGT GGCGGAAGTG TGATGTTGCA AGTGTGGCGG         150

AACACATGTA AGCGACGGAT GTGGCAAAAG TGACGTTTTT GGTGTGCGCC         200

GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC GGATGTTGTA         250

GTAAATTTGG GCGTAACCGA GTAAGATTTG GCCATTTTCG CGGGAAAACT         300

GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA         350

ATATTTGTCT AGGGAGATCT GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC         400

CCGGGCAAAG CCCGGGCGTC GGGCGACCTT TGGTCGCCCG GCCTCAGTGA         450

GCGAGCGAGC GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC         500

TTGTAGTTAA TGATTAACCC GCCATGCTAC TTATCTACAA TTCGAGCTTG         550

CATGCCTGCA GGTCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA         600

CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT         650

AGTAACGCCA ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC         700

GGTAAACTGC CCACTTGGCA GTACATCAAG TGTATCATAT GCCAAGTACG         750

CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCTGGC ATTATGCCCA         800

GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC TACGTATTAG         850

TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT         900

GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC CCATTGACGT         950

CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC        1000
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GTAACAACTC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG       1050

GAGGTCTATA TAAGCAGAGC TCGTTTAGTG AACCGTCAGA TCGCCTGGAG       1100

ACGCCATCCA CGCTGTTTTG ACCTCCATAG AAGACACCGG GACCGATCCA       1150

GCCTCCGGAC TCTAGAGGAT CCGGTACTCG AGGAACTGAA AAACCAGAAA       1200

GTTAACTGGT AAGTTTAGTC TTTTTGTCTT TTATTTCAGG TCCCGGATCC       1250

GGTGGTGGTG CAAATCAAAG AACTGCTCCT CAGTGGATGT TGCCTTTACT       1300

TCTAGGCCTG TACGGAAGTG TTACTTCTGC TCTAAAAGCT GCGGAATTGT       1350

ACCCGCGGCC GCAATTCCCG GGGATCGAAA GAGCCTGCTA AAGCAAAAAA       1400

GAAGTCACCA TGTCGTTTAC TTTGACCAAC AAGAACGTGA TTTTCGTTGC       1450

CGGTCTGGGA GGCATTGGTC TGGACACCAG CAAGGAGCTG CTCAAGCGCG       1500

ATCCCGTCGT TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA       1550

CTTAATCGCC TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA       1600

AGAGGCCCGC ACCGATCGCC CTTCCCAACA GTTGCGCAGC CTGAATGGCG       1650

AATGGCGCTT TGCCTGGTTT CCGGCACCAG AAGCGGTGCC GGAAAGCTGG       1700

CTGGAGTGCG ATCTTCCTGA GGCCGATACT GTCGTCGTCC CCTCAAACTG       1750

GCAGATGCAC GGTTACGATG CGCCCATCTA CACCAACGTA ACCTATCCCA       1800

TTACGGTCAA TCCGCCGTTT GTTCCCACGG AGAATCCGAC GGGTTGTTAC       1850

TCGCTCACAT TTAATGTTGA TGAAAGCTGG CTACAGGAAG GCCAGACGCG       1900

AATTATTTTT GATGGCGTTA ACTCGGCGTT TCATCTGTGG TGCAACGGGC       1950

GCTGGGTCGG TTACGGCCAG GACAGTCGTT TGCCGTCTGA ATTTGACCTG       2000

AGCGCATTTT TACGCGCCGG AGAAAACCGC CTCGCGGTGA TGGTGCTGCG       2050

TTGGAGTGAC GGCAGTTATC TGGAAGATCA GGATATGTGG CGGATGAGCG       2100

GCATTTTCCG TGACGTCTCG TTGCTGCATA AACCGACTAC ACAAATCAGC       2150

GATTTCCATG TTGCCACTCG CTTTAATGAT GATTTCAGCC GCGCTGTACT       2200

GGAGGCTGAA GTTCAGATGT GCGGCGAGTT GCGTGACTAC CTACGGGTAA       2250

CAGTTTCTTT ATGGCAGGGT GAAACGCAGG TCGCCAGCGG CACCGCGCCT       2300
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51

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TTCGGCGGTG  AAATTATCGA  TGAGCGTGGT  GGTTATGCCG  ATCGCGTCAC    2350

ACTACGTCTG  AACGTCGAAA  ACCCGAAACT  GTGGAGCGCC  GAAATCCCGA    2400

ATCTCTATCG  TGCGGTGGTT  GAACTGCACA  CCGCCGACGG  CACGCTGATT    2450

GAAGCAGAAG  CCTGCGATGT  CGGTTTCCGC  GAGGTGCGGA  TTGAAAATGG    2500

TCTGCTGCTG  CTGAACGGCA  AGCCGTTGCT  GATTCGAGGC  GTTAACCGTC    2550

ACGAGCATCA  TCCTCTGCAT  GGTCAGGTCA  TGGATGAGCA  GACGATGGTG    2600

CAGGATATCC  TGCTGATGAA  GCAGAACAAC  TTTAACGCCG  TGCGCTGTTC    2650

GCATTATCCG  AACCATCCGC  TGTGGTACAC  GCTGTGCGAC  CGCTACGGCC    2700

TGTATGTGGT  GGATGAAGCC  AATATTGAAA  CCCACGGCAT  GGTGCCAATG    2750

AATCGTCTGA  CCGATGATCC  GCGCTGGCTA  CCGGCGATGA  GCGAACGCGT    2800

AACGCGAATG  GTGCAGCGCG  ATCGTAATCA  CCCGAGTGTG  ATCATCTGGT    2850

CGCTGGGGAA  TGAATCAGGC  CACGGCGCTA  ATCACGACGC  GCTGTATCGC    2900

TGGATCAAAT  CTGTCGATCC  TTCCCGCCCG  GTGCAGTATG  AAGGCGGCGG    2950

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CGCCGGGCAA  CTCTGGCTCA  CAGTACGCGT  AGTGCAACCG  AACGCGACCG    3600
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52

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AAACAACTGC  TGACGCCGCT  GCGCGATCAG  TTCACCCGTG  CACCGCTGGA     3850

TAACGACATT  GGCGTAAGTG  AAGCGACCCG  CATTGACCCT  AACGCCTGGG     3900

TCGAACGCTG  GAAGGCGGCG  GGCCATTACC  AGGCCGAAGC  AGCGTTGTTG     3950

CAGTGCACGG  CAGATACACT  TGCTGATGCG  GTGCTGATTA  CGACCGCTCA     4000

CGCGTGGCAG  CATCAGGGGA  AAACCTTATT  TATCAGCCGG  AAAACCTACC     4050

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CATTATGTAC  TATTTAAAAA  ACACAAACTT  TTGGATGTTC  GGTTTATTCT     4650

TTTTCTTTTA  CTTTTTTATC  ATGGGAGCCT  ACTTCCCGTT  TTTCCCGATT     4700

TGGCTACATG  ACATCAACCA  TATCAGCAAA  AGTGATACGG  GTATTATTTT     4750

TGCCGCTATT  TCTCTGTTCT  CGCTATTATT  CCAACCGCTG  TTTGGTCTGC     4800

TTTCTGACAA  ACTCGGCCTC  GACTCTAGGC  GGCCGCGGGG  ATCCAGACAT     4850

GATAAGATAC  ATTGATGAGT  TTGGACAAAC  CACAACTAGA  ATGCAGTGAA     4900
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53

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ATTATAAGCT GCAATAAACA AGTTAACAAC AACAATTGCA TTCATTTTAT        5000

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54

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56

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CGAGGTATGT  AGGCGGTGCT  ACAGAGTTCT  TGAAGTGGTG  GCCTAACTAC    9000

GGCTACACTA  GAAGGACAGT  ATTTGGTATC  TGCGCTCTGC  TGAAGCCAGT    9050

TACCTTCGGA  AAAAGAGTTG  GTAGCTCTTG  ATCCGGCAAA  CAAACCACCG    9100

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TAAAGTATAT  ATGAGTAAAC  TTGGTCTGAC  AGTTACCAAT  GCTTAATCAG    9350

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GACTCCCCGT  CGTGTAGATA  ACTACGATAC  GGGAGGGCTT  ACCATCTGGC    9450

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57

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CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG   10350

ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTTCAA   10398

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4910 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG   50

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CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA   200

CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT   250

CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT   300

TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA   350

ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA   400

GCTTGCATGC CTGCAGGTCG ACTCTAGAGG ATCCGAAAAA ACCTCCCACA   450

CCTCCCCCTG AACCTGAAAC ATAAAATGAA TGCAATTGTT GTTGTTAACT   500

TGTTTATTGC AGCTTATAAT GGTTACAAAT AAAGCAATAG CATCACAAAT   550

TTCACAAATA AAGCATTTTT TTCACTGCAT TCTAGTTGTG GTTTGTCCAA   600

ACTCATCAAT GTATCTTATC ATGTCTGGAT CCCCGCGGCC GCCAAATCAT   650

58

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CAGTGCAAGC GTCTGGCACC TTTCCCATGA TATGATGAAT GTAGCACAGT        750

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TCTCTTTGTT CTGCTCCTGC GTCTGCGACA CGTGCGTCAG ATGCTGCGCC       1950
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59

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GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT    3200

CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC    3250
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60

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AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA      4350

TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG      4400

AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC      4450

TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT      4500

AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA      4550
```

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```
TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC        4600

TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC        4650

AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT        4700

GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT        4750

TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA        4800

AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG        4850

AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG        4900

CCCTTTCGTC                                                    4910
```

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WHAT IS CLAIMED IS.

1.   A recombinant hybrid virus comprising:

(a)   DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(b)   DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(c)   a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(d)   DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(e)   DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell.

2.   The virus according to claim 1 wherein said adenovirus is rendered replication defective by a deletion in all or a part of the E1 gene.

3.   The virus according to claim 2 wherein said adenovirus genome has a deletion in all or a part of the E3 gene.

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4.   The virus according to claim 1 wherein
said adenovirus genome comprising deletions in the DNA
sequences of all or a portion of the adenovirus genes
selected from the group consisting of the E2a gene, the
E4 gene, the late genes L1 through L5, the intermediate
genes IX and IV_a, and a combination thereof.

5.   The virus according to claim 1 wherein
said selected gene is a reporter gene.

6.   The virus according to claim 5 wherein
said reporter gene is selected from the group consisting
of the genes encoding ß-galactosidase, alkaline
phosphatase and green fluorescent protein.

7.   The virus according to claim 1 wherein
said selected gene is a therapeutic gene.

8.   The virus according to claim 7 wherein
said therapeutic gene is selected from the group
consisting of a normal CFTR gene and a normal LDL gene.

9.   The virus according to claim 1 further
comprising the DNA of, or corresponding to, a functional
portion of the genome of an adeno-associated virus *rep*
gene.

10.   A recombinant hybrid vector comprising:
        (a)   DNA sequences of, or corresponding
to, the 5' inverted terminal repeat (ITR) sequences of an
adenovirus and the 5' adenovirus packaging/enhancer
domain;
        (b)   DNA s quences of, or corresponding
to, the 5' adeno-associat d virus (AAV) ITR sequences;

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(c)   a gene encoding a selected protein
operatively linked to regulatory sequences directing
expression of the protein in a target cell *in vivo* or *in
vitro*;

(d)   DNA sequences of, or corresponding
to, the 3' AAV ITR sequences;

(e)   DNA sequences of, or corresponding
to, the 3' adenovirus ITR sequences; and

(d)   plasmid DNA sequences containing
regulatory elements.

11.   A recombinant trans-infection particle
comprising:

(a)   a recombinant hybrid virus comprising:

(i)   DNA sequences of, or corresponding
to, the 5' inverted terminal repeat (ITR) sequences of an
adenovirus and the 5' adenovirus packaging/enhancer
domain;

(ii) DNA sequences of, or corresponding
to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein
operatively linked to regulatory sequences directing
expression of the protein in a target cell *in vivo* or *in
vitro*;

(iv) DNA sequences of, or corresponding
to, the 3' AAV ITR sequences;

(v)   DNA sequences of, or corresponding
to, the 3' adenovirus ITR sequences;

wherein said virus is replication-
defective and is provided with a sufficient portion of
the genome of the adenovirus to permit infection of the
target cell;

(b)   a polycation sequence conjugated to said
hybrid virus; and

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(c)   a plasmid comprising an AAV *rep* gene
operatively linked to regulatory sequences capable of
directing its expression, said plasmid associated with
said polycation sequence.

12.   The trans-infection particle according to
claim 11 wherein said adenovirus DNA lacks the sequence
encoding viral genes.

13.   The trans-infection particle according to
claim 11 wherein said adenovirus genome is rendered
replication-defective by a deletion in all or a part of
the E1 gene.

14.   The particle according to claim 13 wherein
said adenovirus genome has a deletion in all or a part of
the E3 gene.

15.   The particle according to claim 11 wherein
said adenovirus genome has deletions in the DNA sequences
of all or a portion of the adenovirus genes selected from
the group consisting of the E2a gene, the E4 gene, the
late genes L1 through L5, the intermediate genes IX and
IV_a, and a combination thereof.

16.   The particle according to claim 11 wherein
said selected gene is a reporter gene.

17.   The particle according to claim 16 wherein
said reporter gene is selected from the group consisting
of the genes encoding β-galactosidase, alkaline
phosphatase and gr en fluorescent protein.

18.   The particle according to claim 11 wherein
said selected gene is a therapeutic gene.

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19. The particle according to claim 18 wherein said therapeutic gene is selected from the group consisting of a normal CFTR gene and a normal LDL gene.

20. A composition for use in delivering and stably integrating a selected gene into the chromosome of a target cell, said composition comprising

(a) a recombinant hybrid virus comprising:

(i) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(ii) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(iv) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(v) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell; and

(b) a pharmaceutically acceptable carrier.

21. The composition according to claim 20 further comprising a plasmid comprising an AAV *rep* gene under the control of regulatory sequences capable of expressing said *rep* gen in said targ t cell.

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22. The composition according to claim 21 wherein said vector further comprises the DNA of, or corresponding to, at least a functional portion of the genome of an adeno-associated virus *rep* gene.

23. A composition for use in delivering and stably integrating a selected gene into the chromosome of a target cell comprising an effective amount of a recombinant trans-infection particle in a pharmaceutically acceptable carrier, said particle comprising:

(a)   a recombinant hybrid virus comprising:

(i)   DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(ii) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(iv) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(v)   DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell;

(b)   a polycation sequence conjugated to said hybrid virus;

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(c)   a plasmid comprising an AAV *rep* gene operatively linked to regulatory sequences capable of directing its expression, said plasmid associated with said polycation sequence.

24.   A mammalian cell capable of expressing a selected gene introduced therein through transduction of the virus of claim 1, the vector of claim 10, or the trans-infection particle of claim 11.

25.   A method for producing high levels of a recombinant adeno-associated virus comprising the steps of

        (a)   culturing a cell co-transfected with the vector of claim 10 and an optional helper virus in the presence of a plasmid containing an AAV *rep* gene under the control of regulatory sequences capable of expressing said *rep* gene; and

        (b)   isolating from said culture a recombinant AAV.

26.   A method for producing high levels of a recombinant adeno-associated virus comprising the steps of

        (a)   culturing a cell transfected with the particle of claim 11 and

        (b)   isolating from said culture a recombinant AAV.

NheI(7)
BglII(365)
SnaBI(841)
EcoRI(1)
XbaI(1161)
BamHI(1167)
XhoI(1177)
BamHI(1245)
Ad 0-1
ITR
CMV
NotI(1355)

pAd.AV.CMVLacZ
(10.398 bp)
LacZ
ClaI(2316)

FIG. IA

Ad 9-16
ITR

NotI(4829)
BamHI(4839)
BglII(5222)
XbaI(5043)
BamHI(5037)

NheI DIGEST

BamHI(1167)
XhoI(1177)
BamHI(1245)
NotI(1355)
XbaI(1161)
SnaBI(841)
ClaI(2316)
BglII(365)

BamHI(4839)
BamHI(5037)
XbaI(5043)
BglII(5222)
EcoRI(10399)

0
9
16

2 ClaI DIGESTED AD
100 m.u.

FIG. IB
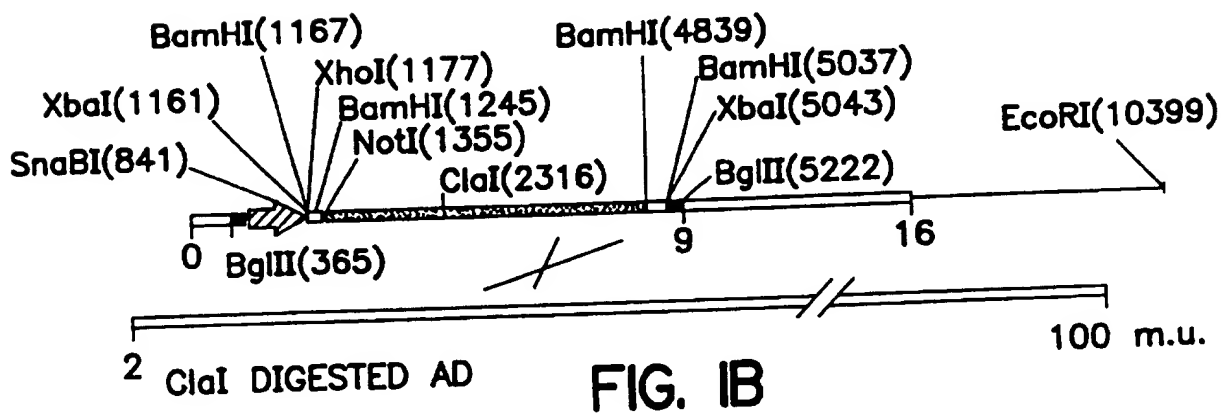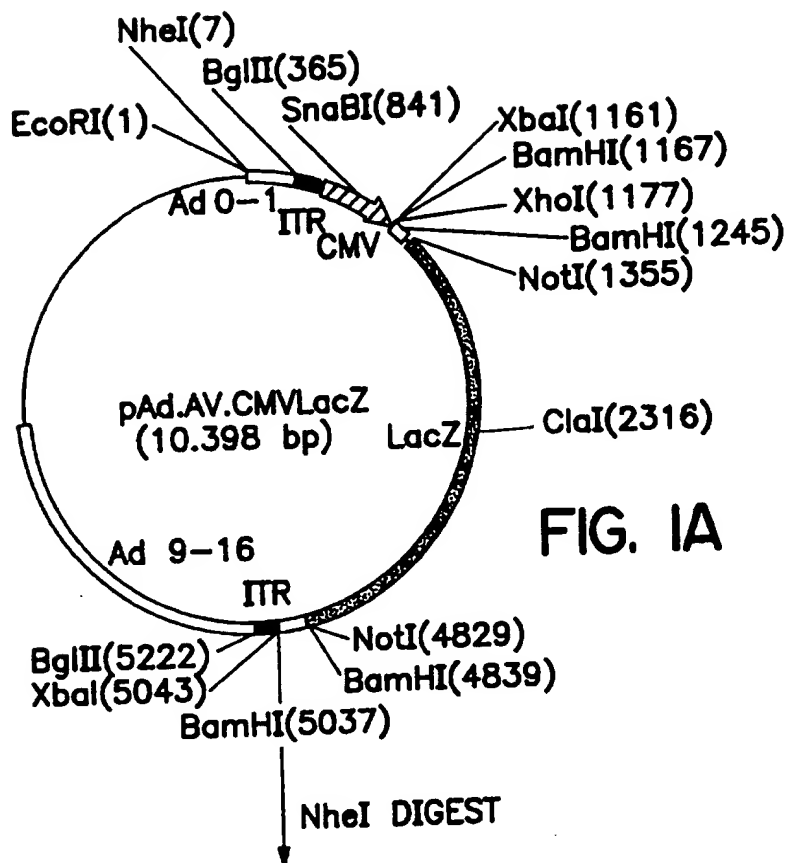
CO-TRANSFECTION INTO
293 CELLS FOLLOWED BY
INTRACELLULAR HOMOLOGOUS
RECOMBINATION.

ClaI(2316)
100 m.u.

0
AV.CMVLacZ
Ad5
Ad5
HYBRID Ad.AV.CMVLacZ

FIG. IC.
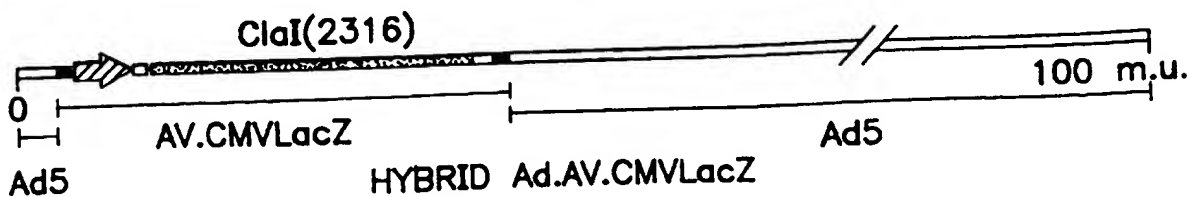
FIGURE 2A

```
GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT ATGATAATGA
                                                                60

GGGGGTGGAG TTTGTGACGT GGCGCGGGGC GTGGGAACGG GGCGGGTGAC GTAGTAGTGT
                                                               120

GGCGGAAGTG TGATGTTGCA AGTGTGGCGG AACACATGTA AGCGACGGAT GTGGCAAAAG
                                                               180

TGACGTTTTT GGTGTGCGCC GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC
                                                               240

GGATGTTGTA GTAAATTTGG GCGTAACCGA GTAAGATTTG GCCATTTTCG CGGGAAAACT
                                                               300

GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA ATATTTGTCT
                                                               360

AGGGAGATCT GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG CCCGGGCGTC
                                                               420

GGGCGACCTT TGGTCGCCCG GCCTCAGTGA GCGAGCGAGC GCGCAGAGAG GGAGTGGCCA
                                                               480

ACTCCATCAC TAGGGGTTCC TTGTAGTTAA TGATTAACCC GCCATGCTAC TTATCTACAA
                                                               540

TTCGAGCTTG CATGCCTGCA GGTCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA
                                                               600

CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT AGTAACGCCA
                                                               660

ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC GGTAAACTGC CCACTTGGCA
                                                               720

GTACATCAAG TGTATCATAT GCCAAGTACG CCCCCTATTG ACGTCAATGA CGGTAAATGG
                                                               780

CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC
                                                               840

TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT
                                                               900

GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC CCATTGACGT CAATGGGAGT
                                                               960

TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC GTAACAACTC CGCCCCATTG
                                                              1020
```

FIGURE 2B

ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA TAAGCAGAGC TCGTTTAGTG
                                                                   1080

AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG AAGACACCGG
                                                                   1140

GACCGATCCA GCCTCCGGAC TCTAGAGGAT CCGGTACTCG AGGAACTGAA AAACCAGAAA
                                                                   1200

GTTAACTGGT AAGTTTAGTC TTTTTGTCTT TTATTTCAGG TCCCGGATCC GGTGGTGGTG
                                                                   1260

CAAATCAAAG AACTGCTCCT CAGTGGATGT TGCCTTTACT TCTAGGCCTG TACGGAAGTG
                                                                   1320

TTACTTCTGC TCTAAAAGCT GCGGAATTGT ACCCGCGGCC GCAATTCCCG GGGATCGAAA
                                                                   1380

GAGCCTGCTA AAGCAAAAAA GAAGTCACCA TGTCGTTTAC TTTGACCAAC AAGAACGTGA
                                                                   1440

TTTTCGTTGC CGGTCTGGGA GGCATTGGTC TGGACACCAG CAAGGAGCTG CTCAAGCGCG
                                                                   1500

ATCCCGTCGT TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA CTTAATCGCC
                                                                   1560

TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA AGAGGCCCGC ACCGATCGCC
                                                                   1620

CTTCCCAACA GTTGCGCAGC CTGAATGGCG AATGGCGCTT TGCCTGGTTT CCGGCACCAG
                                                                   1680

AAGCGGTGCC GGAAAGCTGG CTGGAGTGCG ATCTTCCTGA GGCCGATACT GTCGTCGTCC
                                                                   1740

CCTCAAACTG GCAGATGCAC GGTTACGATG CGCCCATCTA CACCAACGTA ACCTATCCCA
                                                                   1800

TTACGGTCAA TCCGCCGTTT GTTCCCACGG AGAATCCGAC GGGTTGTTAC TCGCTCACAT
                                                                   1860

TTAATGTTGA TGAAAGCTGG CTACAGGAAG GCCAGACGCG AATTATTTTT GATGGCGTTA
                                                                   1920

ACTCGGCGTT TCATCTGTGG TGCAACGGGC GCTGGGTCGG TTACGGCCAG GACAGTCGTT
                                                                   1980

TGCCGTCTGA ATTTGACCTG AGCGCATTTT TACGCGCCGG AGAAAACCGC CTCGCGGTGA
                                                                   2040

FIGURE 2C

TGGTGCTGCG TTGGAGTGAC GGCAGTTATC TGGAAGATCA GGATATGTGG CGGATGAGCG
                                                                    2100

GCATTTTCCG TGACGTCTCG TTGCTGCATA AACCGACTAC ACAAATCAGC GATTTCCATG
                                                                    2160

TTGCCACTCG CTTTAATGAT GATTTCAGCC GCGCTGTACT GGAGGCTGAA GTTCAGATGT
                                                                    2220

GCGGCGAGTT GCGTGACTAC CTACGGGTAA CAGTTTCTTT ATGGCAGGGT GAAACGCAGG
                                                                    2280

TCGCCAGCGG CACCGCGCCT TTCGGCGGTG AAATTATCGA TGAGCGTGGT GGTTATGCCG
                                                                    2340

ATCGCGTCAC ACTACGTCTG AACGTCGAAA ACCCGAAACT GTGGAGCGCC GAAATCCCGA
                                                                    2400

ATCTCTATCG TGCGGTGGTT GAACTGCACA CCGCCGACGG CACGCTGATT GAAGCAGAAG
                                                                    2460

CCTGCGATGT CGGTTTCCGC GAGGTGCGGA TTGAAAATGG TCTGCTGCTG CTGAACGGCA
                                                                    2520

AGCCGTTGCT GATTCGAGGC GTTAACCGTC ACGAGCATCA TCCTCTGCAT GGTCAGGTCA
                                                                    2580

TGGATGAGCA GACGATGGTG CAGGATATCC TGCTGATGAA GCAGAACAAC TTTAACGCCG
                                                                    2640

TGCGCTGTTC GCATTATCCG AACCATCCGC TGTGGTACAC GCTGTGCGAC CGCTACGGCC
                                                                    2700

TGTATGTGGT GGATGAAGCC AATATTGAAA CCCACGGCAT GGTGCCAATG AATCGTCTGA
                                                                    2760

CCGATGATCC GCGCTGGCTA CCGGCGATGA GCGAACGCGT AACGCGAATG GTGCAGCGCG
                                                                    2820

ATCGTAATCA CCCGAGTGTG ATCATCTGGT CGCTGGGGAA TGAATCAGGC CACGGCGCTA
                                                                    2880

ATCACGACGC GCTGTATCGC TGGATCAAAT CTGTCGATCC TTCCCGCCCG GTGCAGTATG
                                                                    2940

AAGGCGGCGG AGCCGACACC ACGGCCACCG ATATTATTTG CCCGATGTAC GCGCGCGTGG
                                                                    3000

ATGAAGACCA GCCCTTCCCG GCTGTGCCGA AATGGTCCAT CAAAAAATGG CTTTCGCTAC
                                                                    3060

FIGURE 2D

CTGGAGAGAC GCGCCCGCTG ATCCTTTGCG AATACGCCCA CGCGATGGGT AACAGTCTTG
3120

GCGGTTTCGC TAAATACTGG CAGGCGTTTC GTCAGTATCC CCGTTTACAG GGCGGCTTCG
3180

TCTGGGACTG GGTGGATCAG TCGCTGATTA AATATGATGA AAACGGCAAC CCGTGGTCGG
3240

CTTACGGCGG TGATTTTGGC GATACGCCGA ACGATCGCCA GTTCTGTATG AACGGTCTGG
3300

TCTTTGCCGA CCGCACGCCG CATCCAGCGC TGACGGAAGC AAAACACCAG CAGCAGTTTT
3360

TCCAGTTCCG TTTATCCGGG CAAACCATCG AAGTGACCAG CGAATACCTG TTCCGTCATA
3420

GCGATAACGA GCTCCTGCAC TGGATGGTGG CGCTGGATGG TAAGCCGCTG GCAAGCGGTG
3480

AAGTGCCTCT GGATGTCGCT CCACAAGGTA AACAGTTGAT TGAACTGCCT GAACTACCGC
3540

AGCCGGAGAG CGCCGGGCAA CTCTGGCTCA CAGTACGCGT AGTGCAACCG AACGCGACCG
3600

CATGGTCAGA AGCCGGGCAC ATCAGCGCCT GGCAGCAGTG GCGTCTGGCG GAAAACCTCA
3660

GTGTGACGCT CCCCGCCGCG TCCCACGCCA TCCCGCATCT GACCACCAGC GAAATGGATT
3720

TTTGCATCGA GCTGGGTAAT AAGCGTTGGC AATTTAACCG CCAGTCAGGC TTTCTTTCAC
3780

AGATGTGGAT TGGCGATAAA AAACAACTGC TGACGCCGCT GCGCGATCAG TTCACCCGTG
3840

CACCGCTGGA TAACGACATT GGCGTAAGTG AAGCGACCCG CATTGACCCT AACGCCTGGG
3900

TCGAACGCTG GAAGGCGGCG GGCCATTACC AGGCCGAAGC AGCGTTGTTG CAGTGCACGG
3960

CAGATACACT TGCTGATGCG GTGCTGATTA CGACCGCTCA CGCGTGGCAG CATCAGGGGA
4020

AAACCTTATT TATCAGCCGG AAAACCTACC GGATTGATGG TAGTGGTCAA ATGGCGATTA
4080

FIGURE 2E

CCGTTGATGT TGAAGTGGCG AGCGATACAC CGCATCCGGC GCGGATTGGC CTGAACTGCC
                                                              4140

AGCTGGCGCA GGTAGCAGAG CGGGTAAACT GGCTCGGATT AGGGCCGCAA GAAAACTATC
                                                              4200

CCGACCGCCT TACTGCCGCC TGTTTTGACC GCTGGGATCT GCCATTGTCA GACATGTATA
                                                              4260

CCCCGTACGT CTTCCCGAGC GAAAACGGTC TGCGCTGCGG GACGCGCGAA TTGAATTATG
                                                              4320

GCCCACACCA GTGGCGCGGC GACTTCCAGT TCAACATCAG CCGCTACAGT CAACAGCAAC
                                                              4380

TGATGGAAAC CAGCCATCGC CATCTGCTGC ACGCGGAAGA AGGCACATGG CTGAATATCG
                                                              4440

ACGGTTTCCA TATGGGGATT GGTGGCGACG ACTCCTGGAG CCCGTCAGTA TCGGCGGAAT
                                                              4500

TACAGCTGAG CGCCGGTCGC TACCATTACC AGTTGGTCTG GTGTCAAAAA TAATAATAAC
                                                              4560

CGGGCAGGCC ATGTCTGCCC GTATTTCGCG TAAGGAAATC CATTATGTAC TATTTAAAAA
                                                              4620

ACACAAACTT TTGGATGTTC GGTTTATTCT TTTTCTTTTA CTTTTTTATC ATGGGAGCCT
                                                              4680

ACTTCCCGTT TTTCCCGATT TGGCTACATG ACATCAACCA TATCAGCAAA AGTGATACGG
                                                              4740

GTATTATTTT TGCCGCTATT TCTCTGTTCT CGCTATTATT CCAACCGCTG TTTGGTCTGC
                                                              4800

TTTCTGACAA ACTCGGCCTC GACTCTAGGC GGCCGCGGGG ATCCAGACAT GATAAGATAC
                                                              4860

ATTGATGAGT TTGGACAAAC CACAACTAGA ATGCAGTGAA AAAAATGCTT TATTTGTGAA
                                                              4920

ATTTGTGATG CTATTGCTTT ATTTGTAACC ATTATAAGCT GCAATAAACA AGTTAACAAC
                                                              4980

AACAATTGCA TTCATTTTAT GTTTCAGGTT CAGGGGGAGG TGTGGGAGGT TTTTTCGGAT
                                                              5040

CCTCTAGAGT CGAGTAGATA AGTAGCATGG CGGGTTAATC ATTAACTACA AGGAACCCCT
                                                              5100

FIGURE 2F

```
AGTGATGGAG TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG CCGGGCGACC
                                                                5160

AAAGGTCGCC CGACGCCCGG GCTTTGCCCG GGCGGCCTCA GTGAGCGAGC GAGCGCGCAG
                                                                5220

CAGATCTGGA AGGTGCTGAG GTACGATGAG ACCCGCACCA GGTGCAGACC CTGCGAGTGT
                                                                5280

GGCGGTAAAC ATATTAGGAA CCAGCCTGTG ATGCTGGATG TGACCGAGGA GCTGAGGCCC
                                                                5340

GATCACTTGG TGCTGGCCTG CACCCGCGCT GAGTTTGGCT CTAGCGATGA AGATACAGAT
                                                                5400

TGAGGTACTG AAATGTGTGG GCGTGGCTTA AGGGTGGGAA AGAATATATA AGGTGGGGGT
                                                                5460

CTTATGTAGT TTTGTATCTG TTTTGCAGCA GCCGCCGCCG CCATGAGCAC CAACTCGTTT
                                                                5520

GATGGAAGCA TTGTGAGCTC ATATTTGACA ACGCGCATGC CCCCATGGGC CGGGGTGCGT
                                                                5580

CAGAATGTGA TGGGCTCCAG CATTGATGGT CGCCCCGTCC TGCCCGCAAA CTCTACTACC
                                                                5640

TTGACCTACG AGACCGTGTC TGGAACGCCG TTGGAGACTG CAGCCTCCGC CGCCGCTTCA
                                                                5700

GCCGCTGCAG CCACCGCCCG CGGGATTGTG ACTGACTTTG CTTTCCTGAG CCCGCTTGCA
                                                                5760

AGCAGTGCAG CTTCCCGTTC ATCCGCCCGC GATGACAAGT TGACGGCTCT TTTGGCACAA
                                                                5820

TTGGATTCTT TGACCCGGGA ACTTAATGTC GTTTCTCAGC AGCTGTTGGA TCTGCGCCAG
                                                                5880

CAGGTTTCTG CCCTGAAGGC TTCCTCCCCT CCCAATGCGG TTTAAAACAT AAATAAAAAA
                                                                5940

CCAGACTCTG TTTGGATTTG GATCAAGCAA GTGTCTTGCT GTCTTTATTT AGGGGTTTTG
                                                                6000

CGCGCGCGGT AGGCCCGGGA CCAGCGGTCT CGGTCGTTGA GGGTCCTGTG TATTTTTTCC
                                                                6060

AGGACGTGGT AAAGGTGACT CTGGATGTTC AGATACATGG GCATAAGCCC GTCTCTGGGG
                                                                6120
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FIGURE 2G

TGGAGGTAGC ACCACTGCAG AGCTTCATGC TGCGGGGTGG TGTTGTAGAT GATCCAGTCG
                                                                    6180

TAGCAGGAGC GCTGGGCGTG GTGCCTAAAA ATGTCTTTCA GTAGCAAGCT GATTGCCAGG
                                                                    6240

GGCAGGCCCT TGGTGTAAGT GTTTACAAAG CGGTTAAGCT GGGATGGGTG CATACGTGGG
                                                                    6300

GATATGAGAT GCATCTTGGA CTGTATTTTT AGGTTGGCTA TGTTCCCAGC CATATCCCTC
                                                                    6360

CGGGGATTCA TGTTGTGCAG AACCACCAGC ACAGTGTATC CGGTGCACTT GGGAAATTTG
                                                                    6420

TCATGTAGCT TAGAAGGAAA TGCGTGGAAG AACTTGGAGA CGCCCTTGTG ACCTCCAAGA
                                                                    6480

TTTTCCATGC ATTCGTCCAT AATGATGGCA ATGGGCCCAC GGGCGGCGGC CTGGGCGAAG
                                                                    6540

ATATTTCTGG GATCACTAAC GTCATAGTTG TGTTCCAGGA TGAGATCGTC ATAGGCCATT
                                                                    6600

TTTACAAAGC GCGGGCGGAG GGTGCCAGAC TGCGGTATAA TGGTTCCATC CGGCCCAGGG
                                                                    6660

GCGTAGTTAC CCTCACAGAT TTGCATTTCC CACGCTTTGA GTTCAGATGG GGGGATCATG
                                                                    6720

TCTACCTGCG GGGCGATGAA GAAAACGGTT TCCGGGGTAG GGGAGATCAG CTGGGAAGAA
                                                                    6780

AGCAGGTTCC TGAGCAGCTG CGACTTACCG CAGCCGGTGG GCCCGTAAAT CACACCTATT
                                                                    6840

ACCGGGTGCA ACTGGTAGTT AAGAGAGCTG CAGCTGCCGT CATCCCTGAG CAGGGGGGCC
                                                                    6900

ACTTCGTTAA GCATGTCCCT GACTCGCATG TTTTCCCTGA CCAAATCCGC CAGAAGGCGC
                                                                    6960

TCGCCGCCCA GCGATAGCAG TTCTTGCAAG GAAGCAAAGT TTTTCAACGG TTTGAGACCG
                                                                    7020

TCCGCCGTAG GCATGCTTTT GAGCGTTTGA CCAAGCAGTT CCAGGCGGTC CCACAGCTCG
                                                                    7080

GTCACCTGCT CTACGGCATC TCGATCCAGC ATATCTCCTC GTTTCGCGGG TTGGGGCGGC
                                                                    7140

FIGURE 2H

TTTCGCTGTA CGGCAGTAGT CGGTGCTCGT CCAGACGGGC CAGGGTCATG TCTTTCCACG
                                                                    7200

GGCGCAGGGT CCTCGTCAGC GTAGTCTGGG TCACGGTGAA GGGGTGCGCT CCGGGCTGCG
                                                                    7260

CGCTGGCCAG GGTGCGCTTG AGGCTGGTCC TGCTGGTGCT GAAGCGCTGC CGGTCTTCGC
                                                                    7320

CCTGCGCGTC GGCCAGGTAG CATTTGACCA TGGTGTCATA GTCCAGCCCC TCCGCGGCGT
                                                                    7380

GGCCCTTGGC GCGCAGCTTG CCCTTGGAGG AGGCGCCGCA CGAGGGGCAG TGCAGACTTT
                                                                    7440

TGAGGGCGTA GAGCTTGGGC GCGAGAAATA CCGATTCCGG GGAGTAGGCA TCCGCGCCGC
                                                                    7500

AGGCCCCGCA GACGGTCTCG CATTCCACGA GCCAGGTGAG CTCTGGCCGT TCGGGGTCAA
                                                                    7560

AAACCAGGTT TCCCCCATGC TTTTTGATGC GTTTCTTACC TCTGGTTTCC ATGAGCCGGT
                                                                    7620

GTCCACGCTC GGTGACGAAA AGGCTGTCCG TGTCCCCGTA TACAGACTTG AGAGGCCTGT
                                                                    7680

CCTCGACCGA TGCCCTTGAG AGCCTTCAAC CCAGTCAGCT CCTTCCGGTG GGCGCGGGGC
                                                                    7740

ATGACTATCG TCGCCGCACT TATGACTGTC TTCTTTATCA TGCAACTCGT AGGACAGGTG
                                                                    7800

CCGGCAGCGC TCTGGGTCAT TTTCGGCGAG GACCGCTTTC GCTGGAGCGC GACGATGATC
                                                                    7860

GGCCTGTCGC TTGCGGTATT CGGAATCTTG CACGCCCTCG CTCAAGCCTT CGTCACTGGT
                                                                    7920

CCCGCCACCA AACGTTTCGG CGAGAAGCAG GCCATTATCG CCGGCATGGC GGCCGACGCG
                                                                    7980

CTGGGCTACG TCTTGCTGGC GTTCGCGACG CGAGGCTGGA TGGCCTTCCC CATTATGATT
                                                                    8040

CTTCTCGCTT CCGGCGGCAT CGGGATGCCC GCGTTGCAGG CCATGCTGTC CAGGCAGGTA
                                                                    8100

GATGACGACC ATCAGGGACA GCTTCAAGGA TCGCTCGCGG CTCTTACCAG CCTAACTTCG
                                                                    8160

FIGURE 2I

ATCACTGGAC CGCTGATCGT CACGGCGATT TATGCCGCCT CGGCGAGCAC ATGGAACGGG
                                                                8220

TTGGCATGGA TTGTAGGCGC CGCCCTATAC CTTGTCTGCC TCCCCGCGTT GCGTCGCGGT
                                                                8280

GCATGGAGCC GGGCCACCTC GACCTGAATG GAAGCCGGCG GCACCTCGCT AACGGATTCA
                                                                8340

CCACTCCAAG AATTGGAGCC AATCAATTCT TGCGGAGAAC TGTGAATGCG CAAACCAACC
                                                                8400

CTTGGCAGAA CATATCCATC GCGTCCGCCA TCTCCAGCAG CCGCACGCGG CGCATCTCGG
                                                                8460

GCAGCGTTGG GTCCTGGCCA CGGGTGCGCA TGATCGTGCT CCTGTCGTTG AGGACCCGGC
                                                                8520

TAGGCTGGCG GGGTTGCCTT ACTGGTTAGC AGAATGAATC ACCGATACGC GAGCGAACGT
                                                                8580

GAAGCGACTG CTGCTGCAAA ACGTCTGCGA CCTGAGCAAC AACATGAATG GTCTTCGGTT
                                                                8640

TCCGTGTTTC GTAAAGTCTG GAAACGCGGA AGTCAGCGCC CTGCACCATT ATGTTCCGGA
                                                                8700

TCTGCATCGC AGGATGCTGC TGGCTACCCT GTGGAACACC TACATCTGTA TTAACGAAGC
                                                                8760

CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG
                                                                8820

GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT
                                                                8880

CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG
                                                                8940

ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC
                                                                9000

GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA
                                                                9060

AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT
                                                                9120

GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC TTTGATCTTT
                                                                9180

FIGURE 2J

TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT GGTCATGAGA
                                                                  9240

TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC
                                                                  9300

TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
                                                                  9360

ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA
                                                                  9420

ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA
                                                                  9480

CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC CGAGCGCAGA
                                                                  9540

AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA
                                                                  9600

GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG
                                                                  9660

GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA
                                                                  9720

GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT
                                                                  9780

GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT
                                                                  9840

CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA
                                                                  9900

TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT
                                                                  9960

ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA
                                                                  10020

AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC
                                                                  10080

AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG
                                                                  10140

CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC
                                                                  10200

FIGURE 2K

CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
                                                                    10260

GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA
                                                                    10320

CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG
                                                                    10380

AGGCCCTTTC GTCTTCAA
          10398

FIG. 3

XbaI(423)
BamHI(429)
BamHI(627)
Sv 40 PA
NotI(635)

PUC18

Amp

PRep 78/52
4910 bp

Rep

BamHI(2653)
XbaI(2547)
P5 PROMOTER
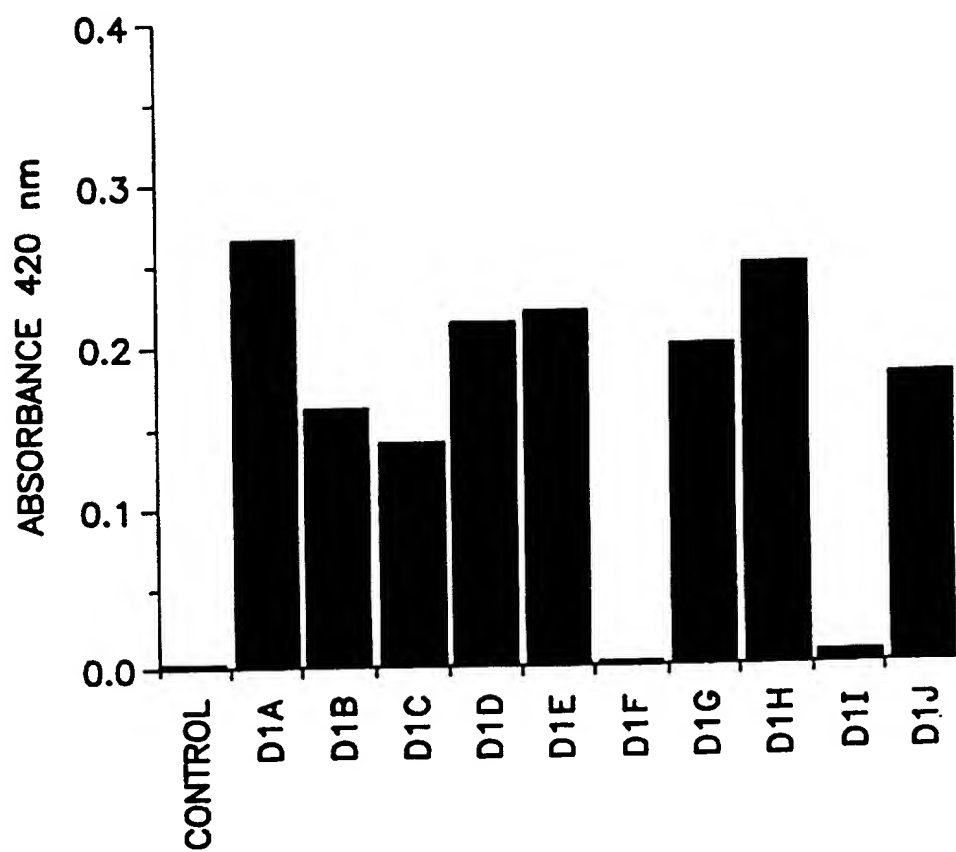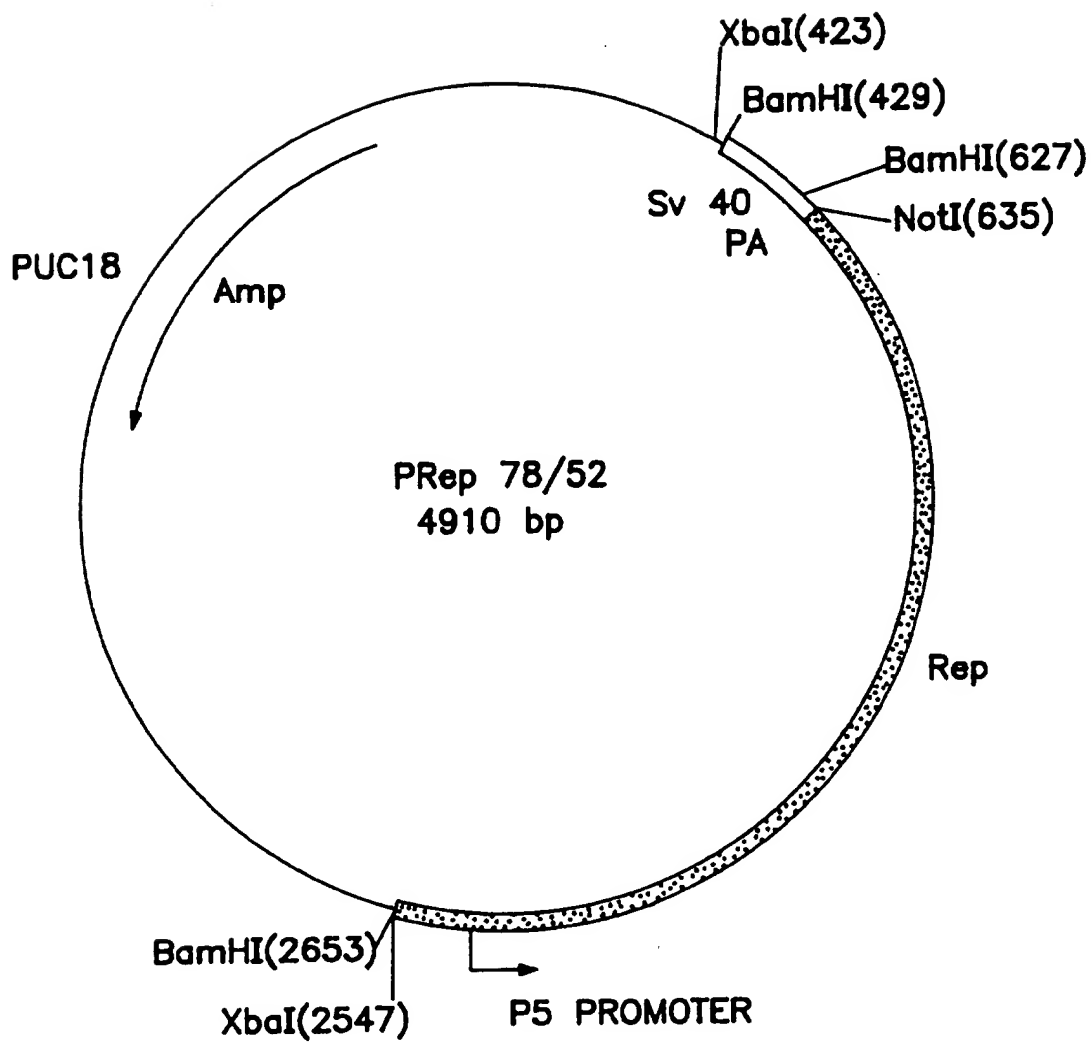
FIG. 4

FIGURE 5A

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA
60

CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG
120

TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC
180

ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC
240

ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT
300

TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT
360

TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA GCTTGCATGC CTGCAGGTCG
420

ACTCTAGAGG ATCCGAAAAA ACCTCCCACA CCTCCCCCTG AACCTGAAAC ATAAAATGAA
480

TGCAATTGTT GTTGTTAACT TGTTTATTGC AGCTTATAAT GGTTACAAAT AAAGCAATAG
540

CATCACAAAT TTCACAAATA AAGCATTTTT TTCACTGCAT TCTAGTTGTG GTTTGTCCAA
600

ACTCATCAAT GTATCTTATC ATGTCTGGAT CCCCGCGGCC GCCAAATCAT TTATTGTTCA
660

AAGATGCAGT CATCCAAATC CACATTGACC AGATCGCAGG CAGTGCAAGC GTCTGGCACC
720

TTTCCCATGA TATGATGAAT GTAGCACAGT TTCTGATACG CCTTTTTGAC GACAGAAACG
780

GGTTGAGATT CTGACACGGG AAAGCACTCT AAACAGTCTT TCTGTCCGTG AGTGAAGCAG
840

ATATTTGAAT TCTGATTCAT TCTCTCGCAT TGTCTGCAGG GAAACAGCAT CAGATTCATG
900

CCCACGTGAC GAGAACATTT GTTTTGGTAC CTGTCTGCGT AGTTGATCGA AGCTTCCGCG
960

TCTGACGTCG ATGGCTGCGC AACTGACTCG CGCACCCGTT TGGGCTCACT TATATCTGCG
1020

FIGURE 5B

TCACTGGGGG CGGGTCTTTT CTTGGCTCCA CCCTTTTTGA CGTAGAATTC ATGCTCCACC
                                                                   1080

TCAACCACGT GATCCTTTGC CCACCGGAAA AAGTCTTTGA CTTCCTGCTT GGTGACCTTC
                                                                   1140

CCAAAGTCAT GATCCAGACG GCGGGTGAGT TCAAATTTGA ACATCCGGTC TTGCAACGGC
                                                                   1200

TGCTGGTGTT CGAAGGTCGT TGAGTTCCCG TCAATCACGG CGCACATGTT GGTGTTGGAG
                                                                   1260

GTGACGATCA CGGGAGTCGG GTCTATCTGG CCGAGGACT TGCATTTCTG GTCCACGCGC
                                                                   1320

ACCTTGCTTC CTCCGAGAAT GGCTTTGGCC GACTCCACGA CCTTGGCGGT CATCTTCCCC
                                                                   1380

TCCTCCCACC AGATCACCAT CTTGTCGACA CAGTCGTTGA AGGGAAAGTT CTCATTGGTC
                                                                   1440

CAGTTTACGC ACCCGTAGAA GGGCACAGTG TGGGCTATGG CCTCCGCGAT GTTGGTCTTC
                                                                   1500

CCGGTAGTTG CAGGCCCAAA CAGCCAGATG GTGTTCCTCT TGCCGAACTT TTTCGTGGCC
                                                                   1560

CATCCCAGAA AGACGGAAGC CGCATATTGG GGATCGTACC CGTTTAGTTC CAAAATTTTA
                                                                   1620

TAAATCCGAT TGCTGGAAAT GTCCTCCACG GGCTGCTGGC CCACCAGGTA GTCGGGGGCG
                                                                   1680

GTTTTAGTCA GGCTCATAAT CTTTCCCGCA TTGTCCAAGG CAGCCTTGAT TTGGGACCGC
                                                                   1740

GAGTTGGAGG CCGCATTGAA GGAGATGTAT GAGGCCTGGT CCTCCTGGAT CCACTGCTTC
                                                                   1800

TCCGAGGTAA TCCCCTTGTC CACGAGCCAC CCGACCAGCT CCATGTACCT GGCTGAAGTT
                                                                   1860

TTTGATCTGA TCACCGGCGC ATCAGAATTG GGATTCTGAT TCTCTTTGTT CTGCTCCTGC
                                                                   1920

GTCTGCGACA CGTGCGTCAG ATGCTGCGCC ACCAACCGTT TACGCTCCGT GAGATTCAAA
                                                                   1980

CAGGCGCTTA AATACTGTTC CATATTAGTC CACGCCCACT GGAGCTCAGG CTGGGTTTTG
                                                                   2040

FIGURE 5C

```
GGGAGCAAGT AATTGGGGAT GTAGCACTCA TCCACCACCT TGTTCCCGCC TCCGGCGCCA
                                                                   2100

TTTCTGGTCT TTGTGACCGC GAACCAGTTT GGCAAAGTCG GCTCGATCCC GCGGTAAATT
                                                                   2160

CTCTGAATCA GTTTTTCGCG AATCTGACTC AGGAAACGTC CCAAAACCAT GGATTTCACC
                                                                   2220

CCGGTGGTTT CCACGAGCAC GTGCATGTGG AAGTAGCTCT CTCCCTTCTC AAAATTGCACA
                                                                   2280

AAGAAAGGG CCTCCGGGGC CTTACTCACA CGGCGCCATT CCGTCAGAAA GTCGCGCTGC
                                                                   2340

AGCTTCTCGG CCACGGTCAG GGGTGCCTGC TCAATCAGAT TCAGATCCAT GTCAGAATCT
                                                                   2400

GGCGGCAACT CCCATTCCTT CTCGGCCACC CAGTTCACAA AGCTGTCAGA AATGCCGGGC
                                                                   2460

AGATGCCCGT CAAGGTCGCT GGGGACCTTA ATCACAATCT CGTAAAACCC CGGCATGGCG
                                                                   2520

GCTGCGCGTT CAAACCTCCC GCTTCAAAAT GGAGACCCTG CGTGCTCACT CGGGCTTAAA
                                                                   2580

TACCCAGCGT GACCACATGG TGTCGCAAAA TGTCGCAAAA CACTCACGTG ACCTCTAATA
                                                                   2640

CAGGACTCTA GAGGATCCCC GGGTACCGAG CTCGAATTCG TAATCATGGT CATAGCTGTT
                                                                   2700

TCCTGTGTGA AATTGTTATC CGCTCACAAT TCCACACAAC ATACGAGCCG GAAGCATAAA
                                                                   2760

GTGTAAAGCC TGGGGTGCCT AATGAGTGAG CTAACTCACA TTAATTGCGT TGCGCTCACT
                                                                   2820

GCCCGCTTTC CAGTCGGGAA ACCTGTCGTG CCAGCTGCAT TAATGAATCG GCCAACGCGC
                                                                   2880

GGGGAGAGGC GGTTTGCGTA TTGGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG
                                                                   2940

CTCGGTCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC
                                                                   3000

CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG
                                                                   3060
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FIGURE 5D

GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA
                                                                    3120

TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA
                                                                    3180

GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG
                                                                    3240

ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCATAGCT CACGCTGTAG
                                                                    3300

GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT
                                                                    3360

TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA
                                                                    3420

CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG
                                                                    3480

CGGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT
                                                                    3540

TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC
                                                                    3600

CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG
                                                                    3660

CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG
                                                                    3720

GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA
                                                                    3780

GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG
                                                                    3840

GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG
                                                                    3900

TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC
                                                                    3960

ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC
                                                                    4020

AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA CTTTATCCGC
                                                                    4080

FIGURE 5E

CTCCATCCAG TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG
                                                                     4140

TTTGCGCAAC GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT
                                                                     4200

GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG
                                                                     4260

CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT
                                                                     4320

GTTATCACTC ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG
                                                                     4380

ATGCTTTTCT GTGACTGGTG AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG
                                                                     4440

ACCGAGTTGC TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT
                                                                     4500

AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT
                                                                     4560

GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC
                                                                     4620

TTTCACCAGC GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT
                                                                     4680

AAGGGCGACA CGGAAATGTT GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT
                                                                     4740

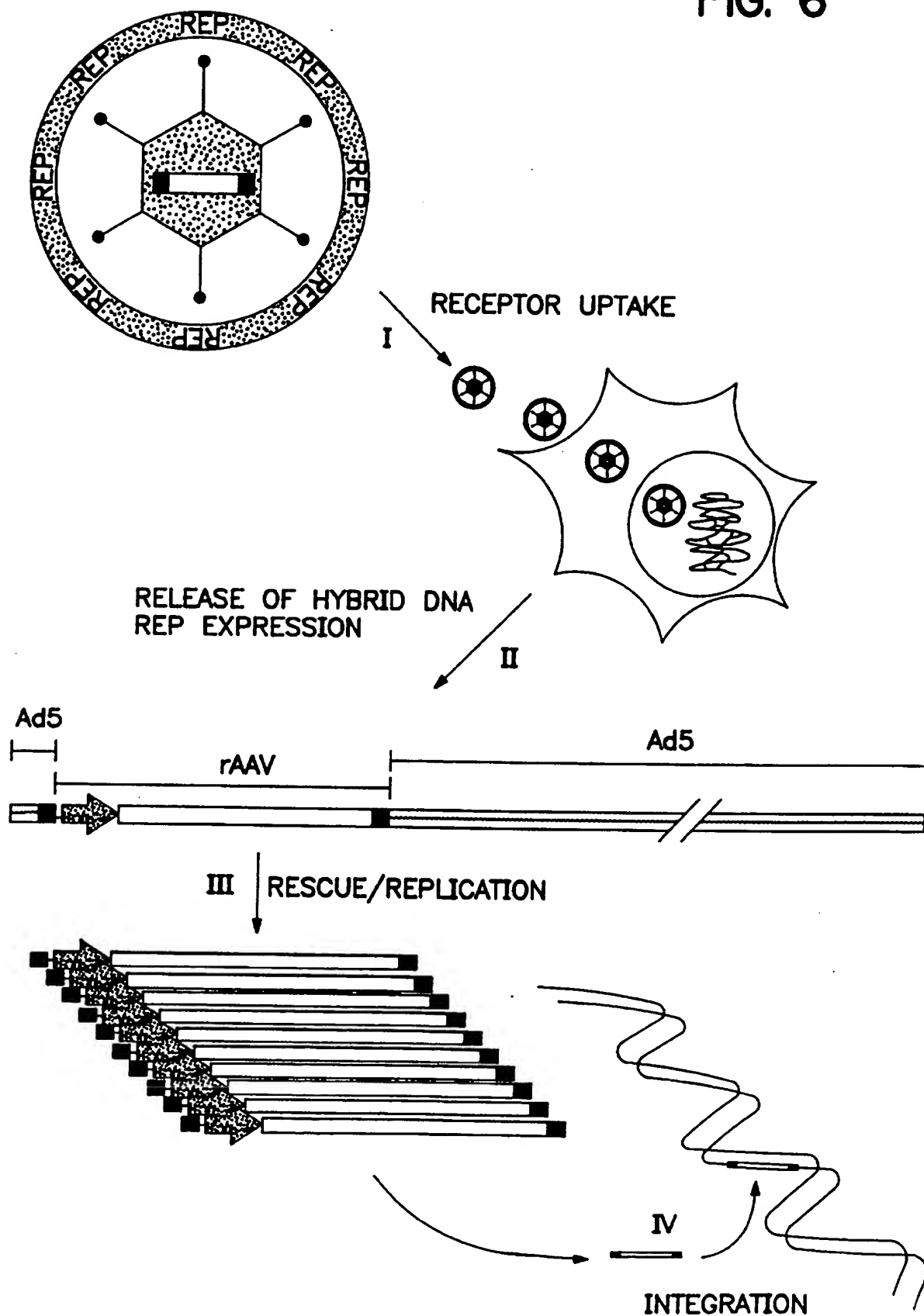TTATCAGGGT TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA
                                                                     4800

AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT
                                                                     4860

TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC
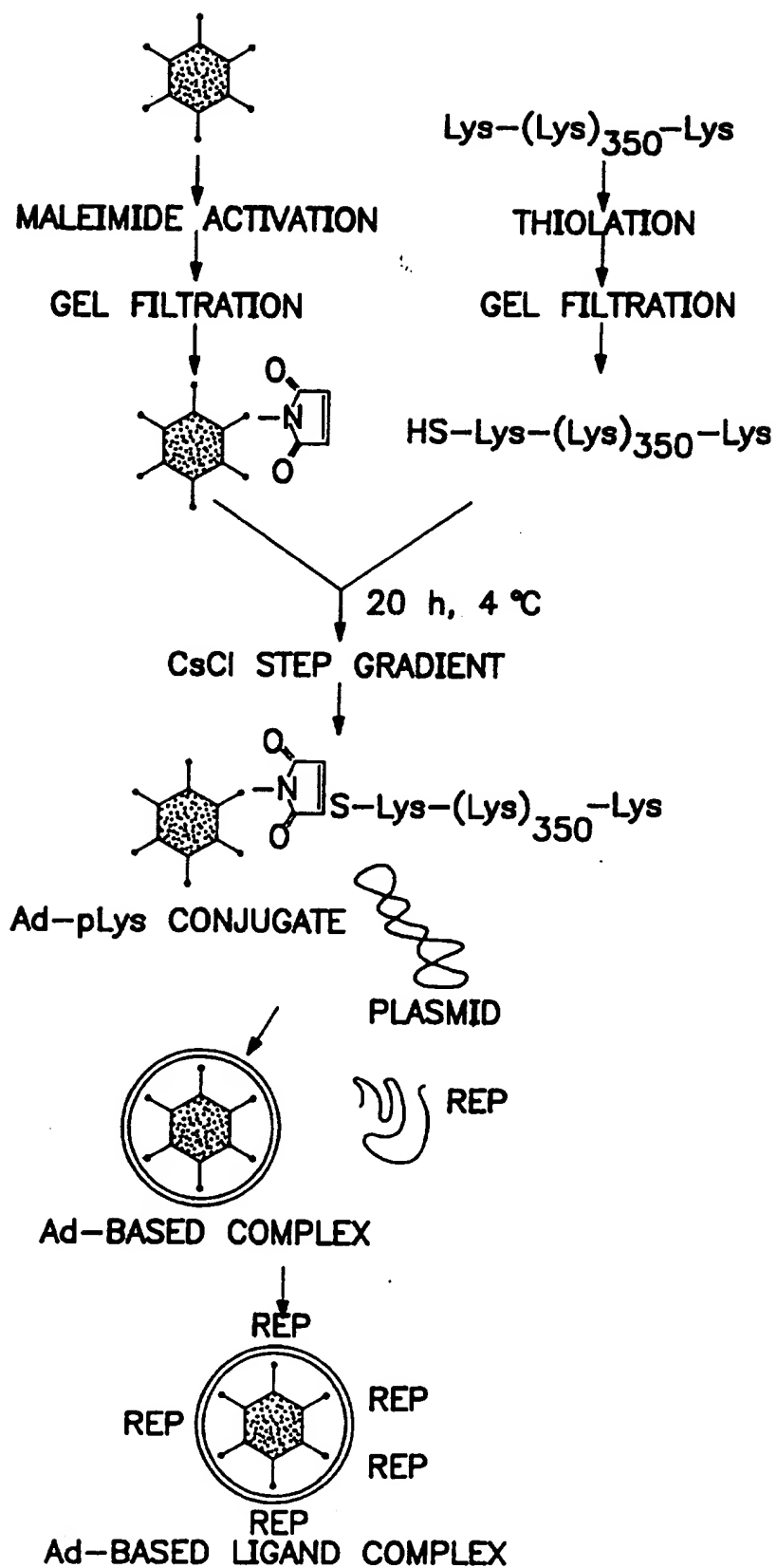   4910

FIG. 6

RECEPTOR UPTAKE

I

RELEASE OF HYBRID DNA
REP EXPRESSION

II

III   RESCUE/REPLICATION

IV

INTEGRATION

FIG. 7